University of Alberta

Polyhydroxybutyrate (PHB) production in transgenic *Arabidopsis thaliana* seeds

by

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©

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Abstract

Polyhydroxybutyrate (PHB) belongs to a class of biodegradable polymers, which has properties similar to conventional plastics. *Ralstronia eutropha* naturally produces PHB using acetyl-CoA. Acetyl-CoA is also abundantly found in developing seeds of higher plants, where oil synthesis takes place. Three genes responsible for PHB synthesis were isolated from *R. eutropha*. Two gene constructs were prepared, driven by seed-specific promoters, to drive PHB synthesis in cytoplasm and leucoplast of developing seeds. The cytosolic construct was introduced into *Arabidopsis thaliana* by *Agrobacterium*-mediated transformation. Homozygous lines with single copy transgenes were developed and characterized at molecular level. Transgenic seeds were analyzed by Gas Chromatography and found to accumulate 0.772 ± 0.006% PHB (dry weight). Furthermore, *phb* genes were over-expressed in *E. coli* and recombinant Phb proteins were purified. Successfully purified Phb proteins were used for immunization into rabbit hosts to produce polyclonal antibodies.

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List of Abbreviations

ACP	acyl carrier protein
Amp^R	Ampicillin resistance
bp	base pair
CaMV35S	cauliflower mosaic virus 35S
CoA	coenzyme A
CTP	Chloroplast transit peptide sequence
DGAT	diacylglycerol acyltransferase
dwt	dry weight
ER	endoplasmic reticulum
fad	fatty acid desaturase
GC	gas chromatography
Gent	Gentamycin
h	hour
HA	3-hydroxyalkanoate
HB	3-hydroxybutyrate
4HB	4-hydroxybutyrate
HD	3-hydroxydecanoate
HH	3-hydroxyhexanoate
HO	3-hydroxyoctanoate
HV	3-hydroxyvalerate
HRP	horseradish peroxidase
Kan^R	Kanamycin resistance
kb	kilo base pair
kDa	kilo Dalton
LB	Luria-Bertani
lcl-	long-chain-length
LSU	rubisco large subunit
mcl-	medium-chain-length

min	minute
Nap	Napin promoter
PAGE	polyacrylamide gel eletrophoresis
PCR	polymerase chain reaction
PHB	poly(3-hydroxybutyrate)
P(HB-HH)	poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
P(HB-HV)	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PDC	pyruvate dehydrogenase complex
PPT	phosphinothricin acetyl transferase
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
scl-	short-chain-length
SDS	sodium dodecyl sulphate
sec	second
SSU	rubisco small subunit
Tet	Tetracycline
TSA	tryptone soy agar
TSB	tryptone soy broth
v/v	volume per volume
w/v	weight per volume
WS	Wassilewskija

Chapter 1 Literature Review

Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants - A review [1]

1.1 Summary

The increasing use of plastics by society and the effects of non-degradable petroleum based plastic wastes on our environment is a growing concern. Polyhydroxyalkanoates (PHAs), macromolecule-polyesters naturally produced by many species of microorganisms, are being considered as a replacement for conventional petroleum-derived plastics. Unlike conventional plastics that take several decades to degrade, PHAs can be completely bio-degraded within a year by a variety of microorganisms that naturally secrete PHA depolymerases. This complete mineralization results in carbon dioxide and water, which return to the environment for use by plants (Madison and Huisman 1999). Many attempts based on various methods have been undertaken for mass production of PHAs. Promising strategies for possible mass production of PHAs involve genetic engineering of microorganisms and plants to introduce pathways for producing the biopolymer. This challenging endeavor requires the expression of several genes along with optimization of PHA synthesis in the new host. Although excellent progress has been made towards PHA production in recombinant hosts, the barriers to obtaining high quality and quantities of PHA at low cost still remain to be solved. The commercially viable production of PHA in crops, however, appears to be a realistic goal for the future (Poirier 2002).

1.2 Introduction

Growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. The accumulation of plastic wastes has become a major concern in terms of the environment. Conventional plastics not only take many decades to be decomposed in nature, but also produce toxins during the process of degradation. For this reason, there is special interest in producing plastics from materials that can be readily eliminated from our biosphere in an "environmentally friendly" fashion. The allure of bioplastic is also linked to diminishing petrochemical reserves. The industrialized world is currently highly dependent on fossil fuels as a source of energy for industrial processes and for the production of structural materials. Fossil fuels are, however, a finite resource and current evidence suggests, based on recent usage trends and the rate of discovery, that utilization rates will outstrip discovery from about 2010 (Zagar 2000). This is a global problem as our economy is still very oil-dependent. The world currently consumes approximately 140 million tons of plastics per annum. Processing of these plastics uses approximately 150 million tons of fossil fuels, which are difficult to substitute. All carbon-based structural materials (e.g., plastics, foams, coating, and adhesives) owe their properties to long arrays of carbon-carbon bonds. The challenge to the world is whether we can substitute the source of these long carbon arrays from a non-sustainable source to a sustainable renewable one.

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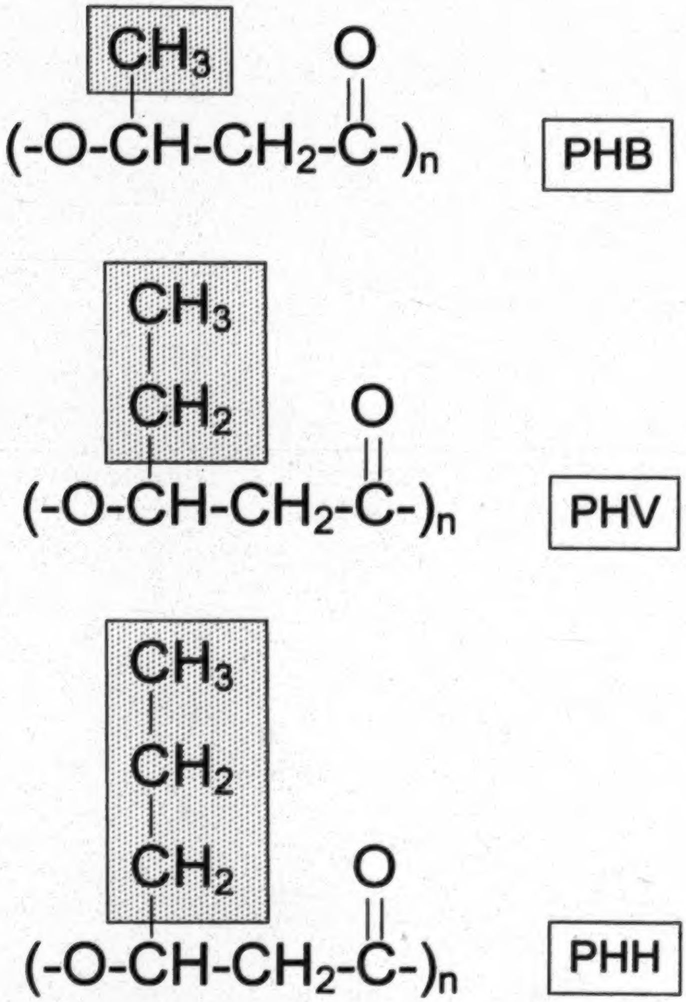
[1] A version of this chapter was submitted as a book chapter to the Woodhead Publishing Limited, Cambridge, U.K.

Bioplastics are natural biopolymers that are synthesized and catabolized by various organisms and these materials do not cause toxic effects in the host and have certain advantages over petroleum-derived plastic (Steinbüchel and Füchtenbusch 1998; Angelova and Hunkeler 1999; Zinn et al. 2001; Williams and Martin 2002). These biopolymers accumulate as storage materials in microbial cells under stress conditions (Barnard and Sander 1989; Sudesh et al. 2000). The most widely produced microbial bioplastics are polyhydroxyalkanoates (PHAs) and their derivatives (Madison and Huisman 1999; Witholt and Kessler 1999). Beijerinck first observed lucent granules of PHA in bacterial cells in 1888 (reported in Chowdhury, 1963). The composition of PHAs was first described by Lemoigne as an unknown material in the form of a homopolyester of the 3-hydroxybutyric acids, called polyhydroxybutyrate (PHB) (Lemoigne 1926, 1927). During the following 30 years, interest in this unknown material was negligible. The first report on functions of PHB appeared in 1958 by Macrae and Wilkinson. They reported the rapid biodegradability of PHB produced by *Bacillus megaterium*, by *B. cereus* and *B. megaterium* itself. From there on, the interest in PHB increased dramatically. In the following years, research on PHB and other forms of PHAs included investigations with other microorganisms and the potential use of these biopolymers was realized (Braunegg et al. 1998). The current review begins with a discussion of the chemical structure and properties of PHAs. This is followed by an examination of PHA synthesis in microorganisms. Finally, numerous strategies are presented showing how genes encoding PHA synthases have been used to produce PHA in plants.

1.3 Monomer composition and physical properties of PHAs

PHAs are composed of 3-hydroxy fatty acid monomers, which form linear, head-to-tail polyester (Figure 1-1). PHA is typically produced as a polymer of 10^3 to 10^4 monomers, which accumulate as inclusions of 0.2-0.5 μm in diameter. These inclusions or granules are synthesized and stored by both gram-positive and gram-negative bacteria without hazardous effects to the hosts (Luengo et al. 2003). PHA accumulation occurs when the cells experience a nutrient imbalance such as excess carbon with limited nitrogen, phosphorus or oxygen (Anderson and Dawes 1990; Steinbüchel 1991; Steinbüchel and Füchtenbusch 1998). The bacteria store the excess nutrients intracellularly by forming insoluble biopolymers from soluble molecules. The biopolymers become mobilized when conditions for normal growth return. The structure, physio-chemical properties, monomer composition and the number and size of the granules vary depending on the organism (Anderson and Dawes 1990; Ha and Cho 2002). In the characterized PHAs, alkyl groups, which occupy the R configuration at the C-3, vary from one carbon (C1) to over 14 carbons (C14) in length. PHAs can be sub-divided into three broad classes according to the size of comprising monomers. PHAs containing up to C5 monomers are classified as short chain length PHAs (scl-PHA). PHAs with C6-C14 and > C14 monomers are classified as medium chain length (mcl-PHA) and long chain length (lcl-PHA) PHAs, respectively (Madison and Huisman 1999). scl-PHAs have properties close to conventional plastics while the mcl-PHAs are regarded as elastomers and rubbers. There are also reports on functional modification of the monomers to improve the properties of the resulting bioplastic, such as the introduction of unsaturated and halogenated branched chains. As well, heteropolymers can be formed by polymerization between more than one kinds of monomer. PHB is the most common type of scl-PHA and this homopolymer of 3-hydroxybutyric acid has been studied most



$$(\text{-O-}\overset{\boxed{CH_3}}{CH}\text{-}CH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-})_n$$

$$(\text{-O-}\overset{\boxed{\overset{CH_3}{\overset{|}{CH_2}}}}{CH}\text{-}CH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-})_n$$

$$(\text{-O-}\overset{\boxed{\overset{CH_3}{\overset{|}{\overset{CH_2}{\overset{|}{CH_2}}}}}}{CH}\text{-}CH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-})_n$$

Figure 1-1. Chemical structure of PHAs
The pendant R groups (shaded boxes) vary in chain length from one carbon (C1) to over 14 carbons (C14). Structures shown here are Polyhydroxybutyrate, PHB, (R = methyl), Plyhydroxyvalerate, PHV (R = ethyl), and polyhydroxyhexanoate, PHH (R = propyl).

extensively. Copolymers of PHA can be formed containing 3-hydroxybutyrate (HB), 3-hydroxyvalerate (HV), 3-hydroxyhexanoate (HH) or 4-hydroxybutyrate (4HB) monomers. Most of the microbes synthesize either scl-PHAs containing primarily 3HB units or mcl-PHAs containing 3-hydroxyoctanoate (HO) and 3-hydroxydecanoate (HD) as the major monomers (Anderson and Dawes 1990; Steinbüchel 1991; Steinbüchel and Schlegel 1991; Lee 1996). Bacteria synthesize a wide range of PHAs and approximately 150 different constituents of PHAs have been identified (Steinbüchel and Valentin 1995).

PHAs extracted from bacterial cells have properties similar to conventional plastics, such as polypropylene (Byrom 1987). PHAs can be degraded at a high rate (3-9 months) by many microorganisms into carbon dioxide and water using their own secreted PHA depolymerases (Jendrossek 2001). They can be produced from renewable resources, are recyclable, and are considered natural materials. These properties make PHAs appropriate for substitution to petrochemical thermoplastics (Poirier 1999a). The large diversity of monomers found in PHAs provides a wide spectrum of polymers with varying physical properties. The homopolymer PHB is a relatively stiff and brittle bioplastic, which is of limited use. PHAs made of longer monomers, such as mcl-PHAs, are typically elastomers and sticky materials, which can also be modified to make rubbers. PHA copolymers composed of primarily HB with a fraction of longer chain monomers, such as HV, HH or HO, are more flexible and tougher plastics. They can be used in a wide variety of products including containers, bottles, razors and materials for food packaging. The latex of PHAs can be used to produce a water-resistant layer for paper, film or cardboard (Hocking and Marchessault 1994). There are manufactures in the U.S. using PHB and copolymer P(HB-HV) as water-proof films on the back of diaper sheets (Martini et al. 1989). This copolymer P(HB-HV), with flexibility and impact resistance, is marketed under the trade name Biopol™ by ICI/Zeneca. Moreover, PHAs are also used to produce fiber materials, such as non-woven fabrics. PHAs with long side chain hydroxyacids have been used in pressure-sensitive adhesive formulations (Yalpani 1993). In addition of their biodegradability, many PHAs are also biocompatible. Their breakdown products are 3-hydroxyacids, which are naturally found in animals. These PHAs can be very useful in many medical applications, such as implants, gauzes, suture filaments, osteosynthetic materials, and a matrix material for slow release drugs.

1.4 PHA synthesis in microorganisms

1.4.1 Genes and enzymes involved in PHA synthesis

Many species of bacteria, which are members of the family Halobactericeae of the Archaea, synthesize PHAs. The most well-known and widely produced form of PHA is PHB. The synthesis of PHB is considered the simplest biosynthetic pathway. The process involves three enzymes and their encoding genes (Figure 1-2). *phaA* gene encodes β-ketothiolase, the first enzyme for the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. The next step is the reduction of acetoacetyl-CoA to *(R)*-3-hydroxybutyryl-CoA catalyzed by the acetoacetyl-CoA reductase. The enzyme is encoded by the *phaB* gene and is NADPH-dependent. The last reaction is the polymerization of *(R)*-3-hydroxybutyryl-CoA monomers catalyzed by PHA synthase, which is encoded by the *phaC* gene. PHA synthase in *Ralstonia eutropha*, formerly known as *Alcaligenes eutrophus*, reacts with a narrow range of substrates, with chains length of C3 - C5. Therefore, PHAs obtained by this pathway contain short-chain-length monomers. All three enzymes for PHB synthesis are located in the cytosol of the cell where PHB accumulation takes place. Apart from PHB, bacteria also synthesize a wide range of other PHAs. A number of PHAs with different C3 to C5 monomers have been produced in several bacteria including *R. eutropha* through alterations in the type and relative quantity of the carbon sources in the growth media. For example, addition of propionic acid or valeric acid in glucose media leads to the production of a random copolymer composed of HB and HV [P(HB-HV)]. In this pathway, the condensation of propionyl-CoA with acetyl-CoA is mediated by a distinct ketothiolase (3-ketothiolase, BtkB) (Figure 1-2). Reduction of 3-ketovaleryl-CoA to *(R)*-3-hydroxyvaleryl-CoA and subsequent polymerization to form P(HB-HV) are catalyzed by the same enzymes involved in PHB synthesis, namely acetoacetyl-CoA reductase and PHA synthase (Figure 1-2). PHA synthases isolated from different bacteria are capable of using a wide range of hydroxyacyl-CoA thioesters as substrates. All known PHA synthases can be classified

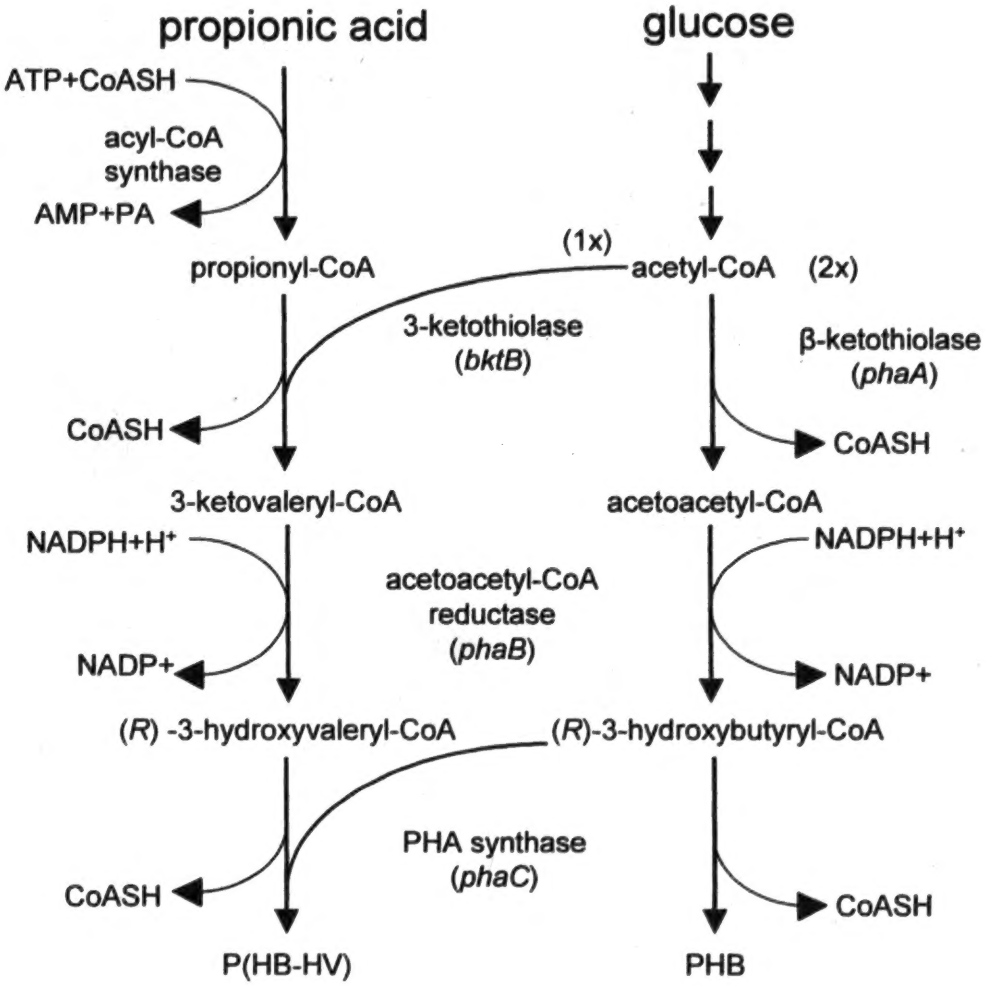

propionic acid
glucose
ATP+CoASH
acyl-CoA synthase
AMP+PA
(1x)
propionyl-CoA
acetyl-CoA
(2x)
3-ketothiolase (*bktB*)
β-ketothiolase (*phaA*)
CoASH
CoASH
3-ketovaleryl-CoA
acetoacetyl-CoA
NADPH+H⁺
NADPH+H⁺
acetoacetyl-CoA reductase (*phaB*)
NADP+
NADP+
(*R*) -3-hydroxyvaleryl-CoA
(*R*)-3-hydroxybutyryl-CoA
PHA synthase (*phaC*)
CoASH
CoASH
P(HB-HV)
PHB

Figure 1-2. PHB and P(HB-HV) biosynthetic pathways in *R. eutropha*
phaA and *bktB* encode β-ketothiolase and 3-ketothiolase, enzymes involved in formation of acetoacetyl-CoA and 3-ketovaleryl-CoA, respectively. *phaB* encodes acetoacetyl-CoA reductase, which reduces both acetoacetyl-CoA and 3-ketovaleryl-CoA to form (*R*)-3-hydroxybutyryl-CoA and (*R*)-3-hydroxyvaleryl-CoA, respectively. *phaC* encodes PHA synthase, which is the last enzyme responsible for polymerization of the monomers (adapted from Poirier 2002).

into four classes according to their substrate specificities and their subunit compositions (Rehm and Steinbüchel 2002; Hai et al. 2004).

Genes encoding key enzymes involved in PHA synthesis have been cloned from several natural producers of the biopolymer. The first *phaA* gene was cloned from *Zoogloea ramigera* using anti-thiolase antibodies (Peoples et al. 1987). Later it was found that the *phaB* gene in this species, and in *Paracoccus denitrificans* and *Rhizobium meliloti*, existed in the same operon, while the *phaC* gene was in a different operon (Figure 1-3b, Tombolini et al. 1995; Yabutani et al. 1995; Lee et al. 1996; Ueda et al. 1996). In *R. eutropha*, *Acinetobacter spp.*, *Alcaligenes latus* and *Pseudomonas acidophila*, the *pha* gene form a *phaCAB* operon, although the three genes are not in the same sequence in these species (Figure1-4a). In some cases the genome carries more than one copy of the operon (Peoples et al. 1989a, b; Schembri et al. 1994; Umeda et al. 1998). In some species including *Chromatium vinosum*, *Thiocystis violacea*, *Thiocapsa pfennigii* and *Synechocystis sp.* PCC 6803, the PHA synthase consists of two sub-units, PhaC and PhaE (Figure 1-3c). This type III synthase mainly catalyzes the synthesis of scl-PHAs, but also catalyzes the polymerization of scl- and mcl-monomers (Hein et al. 1998; Liebergesell and Steinbüchel 1992, 1993; Steinbüchel and Hein 2001).

Additional genes encode other enzymes that indirectly contribute to PHA synthesis. The PHA synthase gene (*phaC*) in *Aeromonas caviae* is flanked by *phaJ*, which encodes enoyl-CoA hydratase (Figure 1-3d). This enzyme catalyzes *(R)*-specific hydration of 2-enoyl-CoA for supplying *(R)*-3-hydroxyacyl-CoA monomers units for PHA synthesis through the fatty acid β-oxidation pathway (Fukui and Doi 1997, Fukui et al. 1998). Unlike *R. eutropha*; *Burkholderia caryophylli*, *P. oleovorans* and *P. aeruginosa* are able to form mcl-PHAs. The *phaC1ZC2D* operon in these organisms contains two *phaC* genes separated by the *phaZ* gene (Figure 1-3e), which encodes a PHA depolymerase (Huisman et al. 1991; Hang et al. 2002). The role of PhaD remains unclear although it seems to be required for PHA formation (Klinke et al. 2000). PHA synthesis in *P. oleovorans* and *P. aeruginosa* directly utilizes intermediates from the fatty acid β-oxidation pathway to form larger molecules of 3-hydroxyacyl-CoA (Lageveen et al.1988). The PHA synthase of *P. oleovorans* can also catalyze the polymerization of a wider

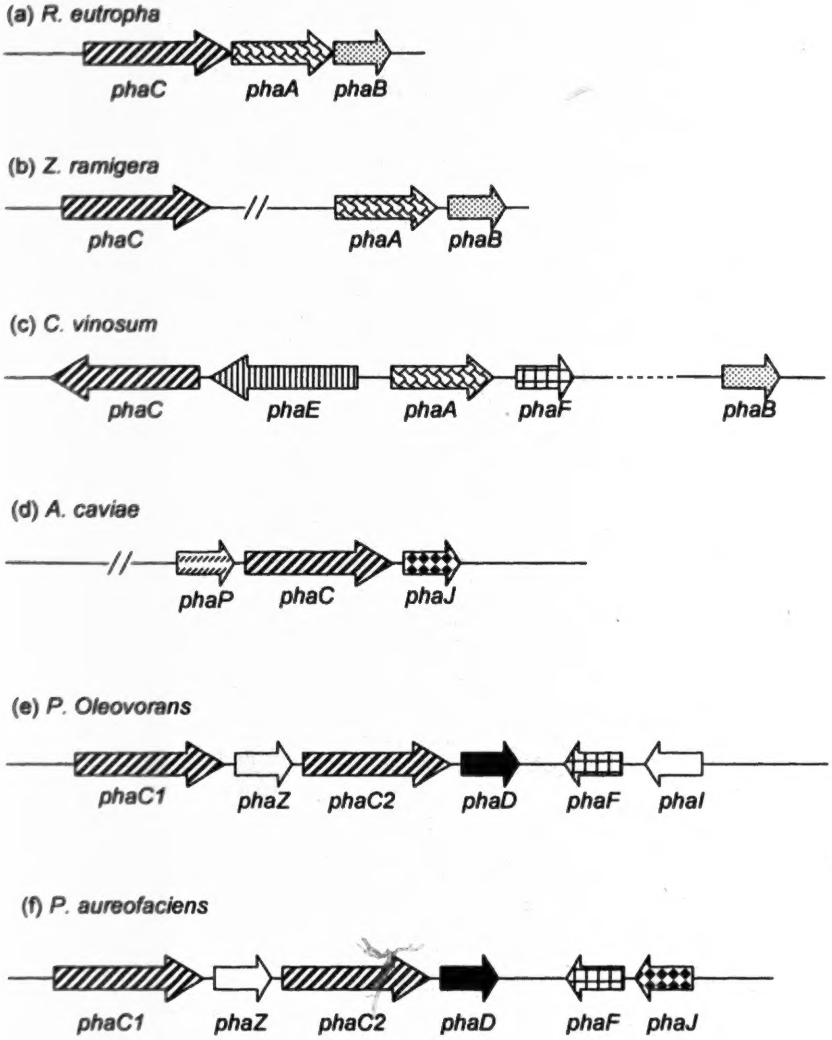

(a) R. eutropha
phaC
phaA
phaB
(b) Z. ramigera
phaC
phaA
phaB
(c) C. vinosum
phaC
phaE
phaA
phaF
phaB
(d) A. caviae
phaP
phaC
phaJ
(e) P. Oleovorans
phaC1
phaZ
phaC2
phaD
phaF
phaI
(f) P. aureofaciens
phaC1
phaZ
phaC2
phaD
phaF
phaJ

Figure 1-3. *pha* gene operons in different microorganisms:
a) a complete *phaCAB* operon of *R. eutropha*; b) interrupted locus of *Z. ramigera*; c) locus with two polymerase subunits, *phaC* and *phaE*, of *C. vinosum*; d) *phaCJ* operon of *A. caviae* for copolymer Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) formation; e) *phaC1ZC2D* operon for medium chain length-PHA formation in *P. oleovorans* with two *phaC* genes (*C1* and *C2*); f) *pha* locus with depolymerase (*phaZ*) between two polymerase subunits in *P. aureofaciens* and *phaF* gene situated downstream of *phaC1ZC2D* operon.

range of monomers, which results in higher molecular weight polymers with better elastic properties. Several microorganisms also carry an additional cluster (*phaFI*) located downstream from the *phaC1ZC2D* operon (Figure 1-3e,f, Nishikawa et al. 2002). PhaI participates in the formation and stabilization of the granules, while PhaF is involved in the stabilization of the granules and acts as a regulator (Prieto et al. 1999).

1.4.2 PHA production in recombinant *Escherichia coli*

In recent years, a combination of genetic engineering and molecular microbiology techniques has been applied to enhance PHA production in microorganisms. Several mutants with phenotypes in PHA synthesis were characterized in order to develop optimal recombinant host strains. Over-expression of *pha* genes in the natural PHA producer, however, resulted in little difference in polymer accumulation. Natural producers, such as *R. eutropha*, are well adapted to PHA accumulation in their cells. *R. eutropha* can store up to 90% of its dry weight (dwt) in PHA granules. Most natural producers, however, take long time to grow during fermentation and extraction of polymers from their cells is difficult. Therefore, these PHA producers are not suitable for industrial production of the biopolymer. On the other hand, although *Escherichia coli* does not naturally produce PHA, this bacterium is considered to be appropriate host for generating higher yields of the biopolymer because of its fast growth and the ease with which it can be lysed. *pha* genes were first introduced into *E. coli* in 1988 by Slater et al. and Schubert et al. PHB granules were formed in recombinant *E. coli* host cells. Even after extensive attempts at maximizing PHB production in non-PHB producing microorganisms, the PHB accumulation level was not as high as what could be obtained with the natural producers of the biopolymer. One of the major obstacles in producing PHB in recombinant organisms is associated with the instability of the introduced *pha* genes. Loss of the plasmid due to metabolic load often limits high yields of the biopolymer. Other parameters have been adjusted to enhance PHB production including increased carbon supply, changes in fermentation temperature, changes in the number of plasmid copies and choice of bacterial strains (Kim et al. 1992; Lee et al. 1994abc, 1995). Growth of the recombinant cells was impaired in many of these studies, especially in nutrient-rich medium (Lee 1994; Wang and Lee 1997). Recombinant *E. coli* cultured under optimal conditions has been shown to accumulate PHB up to 85% of the cell dwt. PHB formed in these *E. coli*, however, were of higher molecular weight than PHB produced by natural producers (Zhang et al. 1994; Kusaka et al. 1997).

1.4.3 Fatty acid β-oxidation and PHA production

The catabolism of fatty acids represents one of the most common pathways to supply hydroxyalkanoate (HA) monomer substrates for PHA synthesis (Sudesh et al. 2000). Intermediates generated by the degradation of alkanoic or fatty acids via β-oxidation (Fad pathway) can provide hydroxyalkanoyl-CoA (HA-CoA) substrate for mcl-PHAs. This pathway is found in several bacteria, such as *P. oleovorans* and *P. fragii*, which can synthesize mcl-PHA from alkanoic acids or fatty acids. In these bacteria, the monomer composition of PHAs produced is directly related to the substrate used for growth and usually has monomers that are 2n (n>=0) carbons shorter than the substrates (Lageveen et al. 1988). Although wild type *E. coli* cannot accumulate PHA, its fatty acid metabolic pathways have been utilized to provide mcl-HA-CoA precursors for PHA accumulation in engineered *E. coli* cells. The intermediates of fatty acid β-oxidation pathways including enoyl-CoA, 3-ketoacyl-CoA and *(S)*-3-hydroxyacyl-CoA, can serve as precursors of mcl- *(R)*-3-hydroxyacyl-CoA, which is used directly in mcl-PHA synthesis (Figure 1-4). Therefore, it was possible to synthesize mcl-PHA in *E. coli* cells, which are defective in the β-oxidation pathway (*fadA* and *fadB* mutants, accumulate β-oxidation intermediates), by introducing the *Pseudomonas* mcl-PHA synthase (*phaC*) gene (Langenbach et al. 1997; Qi et al. 1997, 1998; Ren et al. 2000; Park et al. 2002). In these defective *E. coli*, co-expression of other genes, along with PHA synthase, were necessary to convert β-oxidation intermediates to *(R)*-3-hydroxyacyl-CoA, immediate precursor of mcl-PHA (Figure 1-4). Examples of these genes, which were co-expressed with PHA synthase for enhanced production, include *paaF*, *paaG*, *ydbU* from *E. coli* (Park and Lee 2004), *phaJ* from *P. aeruginosa*, *P. putida* and *A. caviae* (Fukui et al. 1999; Park et al. 2001; Fiedler et al. 2002; Tsuge et al. 2002, 2003), *maoC* from *E. coli* (Park and Lee 2003), *yfcX* from *E. coli* (Snell et al. 2002), *fabG* from *P. aeruginosa* and *E. coli*

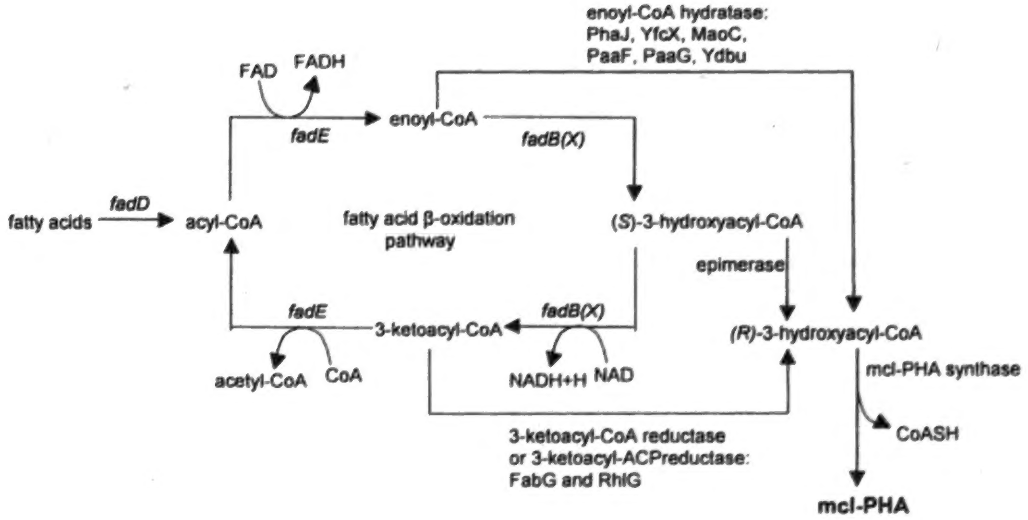

enoyl-CoA hydratase:
PhaJ, YfcX, MaoC,
PaaF, PaaG, Ydbu
FAD
FADH
fadE
enoyl-CoA
fadB(X)
fadD
fatty acids
acyl-CoA
fatty acid β-oxidation pathway
(S)-3-hydroxyacyl-CoA
epimerase
fadE
3-ketoacyl-CoA
fadB(X)
(R)-3-hydroxyacyl-CoA
acetyl-CoA
CoA
NADH+H
NAD
mcl-PHA synthase
CoASH
3-ketoacyl-CoA reductase
or 3-ketoacyl-ACPreductase:
FabG and RhlG
mcl-PHA

Figure 1-4. Fatty acid β-oxidation pathway of *E. coli*
Recombinant *E. coli* with defective *fadA* and *fadB* uses intermediates of β-oxidation, enoyl-CoA, 3-ketoacyl-CoA and *(S)*-3-hydroxyacyl-CoA, as major substrates for mcl-PHA synthesis. Co-expression of medium chain length-PHA synthase with enoyl-CoA hydratase (encoded by *paaF, paaG, ydbU*, *maoC, yfcX* from *E. coli; phaJ* from *P. aeruginosa, P. putida* and *A. caviae*) or 3-ketoacyl-CoA/ACP reductase (encoded by *fabG* from *P. aeruginosa* and *E. coli; rhlG* from *P. aeruginosa*) can enhance PHA production by increasing β-oxidation intermediate pools (adapted from Park and Lee 2003).

(Taguchi et al. 1999; Ren et al. 2000; Park et al. 2002) and *rhlG* from *P. aeruginosa* (Campos-Garcia 1998; Park et al. 2002). Co-expression of other genes of the β-oxidation pathway can significantly enhance PHA production in recombinant *E. coli*. For example, acyl-CoA dehydrogenase of *E. coli*, encoded by *yafH* and *fadE* (Campbell and Cronan 2002), catalyzes the dehydration of acyl-CoA to enoyl-CoA (Figure 1-4) and is considered the rate-limiting step of β-oxidation (Qi et al. 1998). Co-expression of *yafH*, along with *phaC* and *phaJ*, enhanced the supply of enoyl-CoA. With the enhanced precursor supply, the recombinant *E. coli* accumulated four times more scl-mcl-PHA, P(HB-HH) than cells expressing only *phaC* and *phaJ* (Lu et al. 2003). Co-expression of the *E. coli yafH* gene also increased HH content of the P(HB-HH) copolymer produced in recombinant *Aeromonas hydrophila* (Lu et al. 2004). When β-oxidation was inhibited by sodium acrylate in natural producers of scl-PHB, *R. eutropha*, and grown in sodium octanoate as a carbon source, the intermediates of β-oxidation pathway were channeled into mcl-PHAs composed of HH, HO and HB (Green et al. 2002).

A second route of mcl-PHA synthesis in bacteria is through the use of intermediates of fatty acid *de novo* biosynthesis (Fab pathway) that provides HA-CoA monomers (Figure 1-5). In contrast to *P. oleovorans* and *P. fragii* (which use β-oxidation intermediates), *P. aeruginosa* and *P. putida* produce mcl-PHAs when grown on unrelated substrates, such as glucose. This is because *P. oleovorans* and *P. fragii* use fatty acid as carbon source through β-oxidation pathway to produce 3-hydroxyacyl-CoA, substrate of mcl-PHA synthase (Figure 1-4). On the other hand, the fatty acid biosynthesis is the main route for 3-hydroxyacyl-CoA synthesis in *P. aeruginosa* and *P. putida* during growth on a carbon source which is metabolized to acetyl-CoA, like carbohydrate, acetate or ethanol (Figure 1-5). The gene *phaG*, encoding an enzyme linking fatty acid *de novo* biosynthesis and PHA synthesis, has been cloned from *P. putida* (Rehm et al. 1998; Hoffmann et al. 2000a, b). The product of this gene catalyzes the conversion of *(R)*-3-hydroxyacyl-ACP, intermediate of fatty acid biosynthesis pathway, to its corresponding CoA derivative (Figure 1-5). Therefore, expression of *phaG* in *P. oleovorans*, *P. fragii* and *E. coli* gave the recombinant bacteria the novel capacity to synthesize mcl-PHA from carbon sources

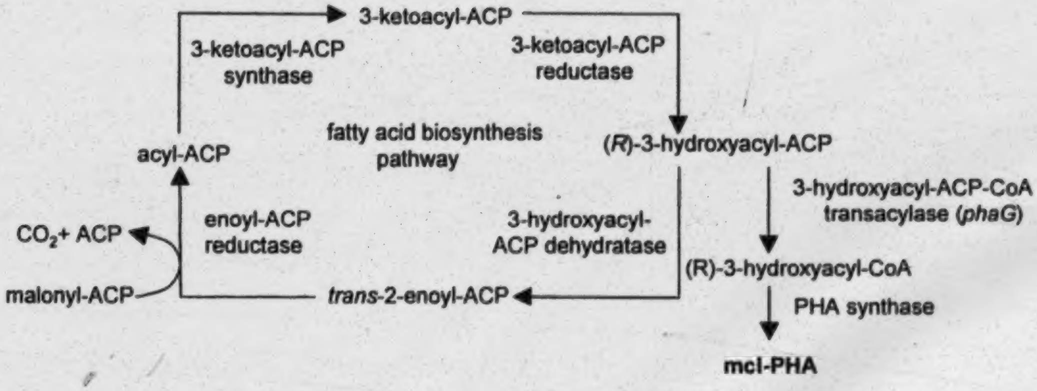

3-ketoacyl-ACP
3-ketoacyl-ACP synthase
3-ketoacyl-ACP reductase
fatty acid biosynthesis pathway
acyl-ACP
(R)-3-hydroxyacyl-ACP
3-hydroxyacyl-ACP-CoA transacylase (phaG)
CO_2+ ACP
enoyl-ACP reductase
3-hydroxyacyl-ACP dehydratase
(R)-3-hydroxyacyl-CoA
malonyl-ACP
trans-2-enoyl-ACP
PHA synthase
mcl-PHA

Figure 1-5. Fatty acid *de novo* biosynthesis (Fab pathway)
P. aeruginosa and *P. putida* use (*R*)-3-hydroxyacyl-CoA monomers from Fab pathway to produce medium chain length (mcl)-PHAs through expression of PhaG and PhaC when grown on carbon sources which is metabolized to acetyl-CoA, like carbohydrate. PhaG acts as a link between Fab pathway and mcl-PHA synthesis by catalyzing (*R*)-3-hydroxyacyl-ACP, intermediate of Fab pathway, to (*R*)-3-hydroxyacyl-CoA, substrate for mcl-PHA.

non-related to HD structure, such as glucose and fructose (Fiedler et al. 2000; Hoffmann et al. 2000b; Zheng et al. 2004).

1.4.4 Engineering of PHA synthases to enhance and change polymer synthesis

A number of studies have focused on engineering PHA synthases in attempts to enhance activity and/or alter substrate specificity for customized production of PHA copolymers in recombinant hosts. Takase et al. (2003) modified the phaC1 gene of *Pseudomonas sp.* through error-prone PCR mutagenesis and site-specific saturation mutagenesis. The gene product of *phaC1* is a type II synthase and, unlike type I enzyme, has a very low specificity for HB units. The engineered enzymes substantially enhanced (400 times) PHB synthesis in recombinant *E. coli*. Amara et al. (2002) also reported enhanced activity of PHA synthase from *A. punctata* modified through genetic engineering. Kichise et al. (2002) achieved enhanced accumulation and changed monomer composition in recombinant *E. coli* through a modification of the *phaC* gene from *A. punctata*. This alteration led to a 6-fold enhancement in accumulation of P(HB-HH). The PhaJ enzyme from *A. caviae* generates *(R)*-3-hydroxyacyl-CoAs with chain length of 4 to 6 carbon atoms from the fatty acid β-oxidation pathway for PHA synthesis (Figure 1-4). Tsuge et al. (2003) examined the structure of this enzyme through x-ray crystallography and identified Ser-62, Leu-65 and Val-130 as amino acid residues that define the depth and width of the acyl-chain-binding pocket. By changing these residues through site-directed mutagenesis of the *phaJ* gene, they created mutants that showed significantly higher specificities towards octenoyl-CoA (C8) than the wild-type enzyme. When the modified gene was introduced into *E. coli* along with *phaC1* of *Pseudomonas sp.* there was an increased incorporation of HO (C8) and HD (C10) into biopolymer.

Nomura et al. (2004a, b) altered 3-ketoacyl-ACP synthase III (FabH) of *E. coli* and PhaC1 of *Pseudomonas sp.* by changing the substrate specificity of the enzymes through site-directed or saturation point mutagenesis of the encoding genes. These engineered genes were then introduced into *E. coli*, along with *phaA* and *phaB*. The cumulative effect of having two monomer-supplying pathways and genetically engineered PHA synthase resulted in accumulation of scl-mcl-PHA copolymer from the non-related carbon source, glucose.

1.4.5 Production of PHA copolymers

scl-PHA homopolymers (C3-C5), such as PHB, form stiff crystalline materials, which are brittle and cannot be extended without breakage. This lack of flexibility limits the range of applications of scl-PHA homopolymers. PHB homopolymer consisting solely of C4 monomer is difficult to process, because it degrades at a temperature slightly above its melting point (De Koning 1995). Polymers consisting of only mcl-PHA (C6-C14) are semi-crystalline thermoplastic elastomers. The mechanical properties of these polymers may be enhanced by reinforcement. Unlike polymers composed solely of either scl- or mcl-monomer units, scl-mcl-PHA copolymers can have a wide range of physical properties, depending on the mole % composition of the different monomers in the copolymer (Matsusaki et al. 2000). scl-mcl-copolymers composed of mostly C4 monomers, with a small amount of C6 monomer, have properties similar to polypropylene (Abe and Doi 2002). scl-mcl-PHA copolymer of HB and HH [P(HB-HH)] is a tough and flexible material. The copolymer of HB and HV [P(HB-HV)] has reduced crystallinity and melting point, leading to improved flexibility, strength and easier processing. Therefore, several laboratories have attempted to synthesize specific scl-mcl-PHA copolymers in bacteria. *pha* genes from different natural producers, such as the *phaC* gene from *Pseudomonas spp*, were introduced into *E. coli* to induce the synthesis of copolymers and mcl-PHA. The *phaC1* gene from *P. oleovorans* in *fadA* and *fadB* strains accumulated mcl-PHAs when grown on C8 to C18 fatty acids, with yield increases achieved by using inducible promoters (Ren et al. 1996). PHA copolymers containing HH, HO and HD were produced in the recombinant *fadB* mutant of *E. coli* by introducing *phaC1* and *phaC2* genes from *P. aeruginosa* and *Burkholderia caryophylli* (Langenbach et al. 1997; Qi et al. 1997; Hang et al. 2002). *E. coli* cells were co-transformed with the *hbcT* gene from *Clostridium kluyveri*, which encodes a 4-hydroxybutyric acid-CoA transferase, and *phaC* from *R. eutropha*. Up to 20% of the cell dwt contained P(4HB) homopolymer when the bacteria were grown in the presence of 4HB. In the presence of glucose P(4HB) homopolymer was produced, while in the absence of glucose, a P(HB-

4HB) copolymer accumulated even though *phaA* and *phaB* genes were absent (Hein et al. 1997). Changing glucose and fatty acid concentration in the medium have also been shown to lead to a change in the monomer composition of PHA copolymer (e.g., Lu et al. 2004). Law et al. (2004) achieved 43% cell dwt P(HB-HV) copolymer production in recombinant *E. coli* through selection of certain strains of the bacteria. By introducing the succinate degradation pathway from *C. kluyveri*, along with *pha* genes from *R. eutropha*, Valentin and Dennis (1997) produced P(HB-4HB) directly from glucose. Other feeding and fermentation strategies were undertaken to optimize the copolymer production levels (Hong et al. 2000; Ahn et al. 2000; Lee and Choi 2001). However, since *E. coli* is not a natural producer of PHAs there were difficulties in optimizing the growth of engineered *E. coli* (Madison and Huisman 1999).

1.5 PHA production in eukaryotic cells

The production of bioplastic in bacteria is limited by its high cost compared to the costs associated with petroleum-derived plastics production. This aspect has been one of driving forces in exploring eukaryotic systems, especially crops, as production hosts. Studies of PHA formation in yeast and insect cells can provide valuable information about how these pathways can be incorporated into plants. Synthesis of PHB has been demonstrated in *Saccharomyces cerevisiae* by expressing the PHB synthase gene from *R. eutropha* (Leaf et al. 1996). PHB accumulation, however, was very low (0.5% of cell dwt), possibly because of insufficient endogenous β-ketoacyl-CoA-thiolase and acetoacetyl-CoA reductase activities. To improve the yield and to synthesize copolymers of PHAs, studies have focused on channeling the intermediates of β-oxidation pathway into PHA assembly. Poirier et al. (2001) introduced a modified *phaC1* gene from *P. aeruginosa* into *S. cerevisiae*. Peroxisomal targeting (PTS1) of the gene product was achieved by developing a construct which resulted in the addition of a 34 amino acid stretch from the carboxylic end of *Brassica napus* isocitrate lysate. When the recombinant yeast cells were grown in media containing fatty acids, they accumulated mcl-PHAs demonstrating that peroxisomal PHA synthase produces PHA in the peroxisomes using 3-hydroxyacyl-CoA intermediates of fatty acid oxidation. In contrast to *S. cerevisiae*, *Pichia pastoris* grows vigorously on fatty acids as a carbon source. Poirier et al. (2002) introduced the above PTS1-modified *P. aeruginosa phaC1* gene into *P. pastoris* and achieved mcl-PHA synthesis in this yeast system with fatty acids in the growth medium. The yield of PHA in the two described studies with yeast systems, however, was low, with accumulations lower than 1% cell dwt.

Marchesini et al. (2003) have explored the possibilities of changing monomer composition of PHA in recombinant yeast cells. The investigators demonstrated that it was possible to alter the PHA monomer composition of mcl-PHAs produced in yeast from the intermediates of the β-oxidation of fatty acids by using modified form of the peroxisomal multifunctional enzyme 2 (MFE-2, encoded by the *fox2* gene). They transformed yeast cells with genes coding for two mutant forms of the 3-hydroxyacyl-CoA dehydrogenase domain of the MFE-2 of *S. cerevisiae*. The mutant MEF-*2*(aΔ) retain a broad activity towards short-, medium- and long-chain (*R)*-3-hydroxyacyl-CoAs, while the mutant MFE-2(bΔ), did not accept short-chain *(R)*-3-hydroxyacyl-CoAs. Expression of MFE-2(bΔ), along with PHA synthase, resulted in a substantial increase in the proportion of the short-chain 3-hydroxyacid monomers at the expense of longer monomers. These transformant yeast cells were inefficient at using short-chain *(R)*-3-hydroacyl-CoAs generated by the β-oxidation cycle, leading to higher levels of these intermediates available to the PHA synthase.

Insect cells have also been studied as a model for PHA production in eukaryotes. The *phaC* gene from *R. eutropha* was successfully expressed in cabbage looper cells and a soluble form of PHB synthase that could be rapidly purified was obtained (Williams et al. 1996a). This was an important observation because expression of PhaC in recombinant *E. coli* (a prokaryote) generally resulted in insoluble, inactive polymerase. In a separate attempt, Williams et al. (1996b) transfected fall armyworm cells with a modified eukaryotic fatty acid synthase, which did not extend fatty acids beyond HB, along with the *phaC* gene from *R. eutropha*. PHB production was achieved in the transfected cells, although the yield was very low (< 1% of cell dwt).

1.6 PHA synthesis in transgenic plants

PHA production in bacteria and yeast requires growth under sterile condition in a costly fermentation process with an external energy source such as electricity. In contrast, PHA production in plant systems is considerably less expensive because the system only relies on water, soil nutrients, atmospheric CO_2 and sunlight. In addition, a plant production system is much more environment friendly. Plants use photosynthetically fixed CO_2 and water to generate the bioplastic, which after disposal is degraded back to CO_2 and water. Synthesis of PHAs in crops is also an excellent way of increasing the value of the crops (Poirier 1999b, Somerville and Bonetta 2001). Since starch and sugar are produced in plants at costs below the cost of commodity plastics, it might be possible to produce PHA at a similar low cost. Unlike the bacterial cell, the plant cell has different subcellular compartments in which PHA synthesis can be metabolically localized. As mentioned earlier, PHB is synthesized in bacteria from acetyl-CoA. This thioester is present in plant cells in the cytosol, plastids, mitochondria and peroxisomes. Therefore, it should be possible to produce PHB in any of these subcellular compartments (Hanley et al. 2000; Moire et al. 2003).

1.6.1 Cytosolic PHA synthesis

Initial work on producing PHB in plants focused on cytosolic expression, which avoids the use of targeting sequences. As well, an endogenous β-ketothiolase, the first critical enzyme in PHB synthesis, occurs naturally in the cytosol of higher plants where it takes part in the synthesis of mevalonate, the precursor to isoprenoids (Figure 1-6). Therefore, PHB synthesis in the cytosol should require only the expression of acetoacetyl-CoA reductase and PHB synthase from bacteria.

The first attempt to produce bioplastic in transgenic plants was carried out with *Arabidopsis thaliana*, a model plant with a relatively small genome and short life cycle that could be easily transformed with *Agrobacterium tumefaceins*. In the pioneering work, Poirier et al. (1992) introduced the *phaB* gene encoding acetoacetyl-CoA reductase and the *phaC* gene encoding PHB synthase from *R. eutropha* into *A. thaliana* plant cells under the control of the constitutive cauliflower mosaic virus 35S (CaMV35S) promoter

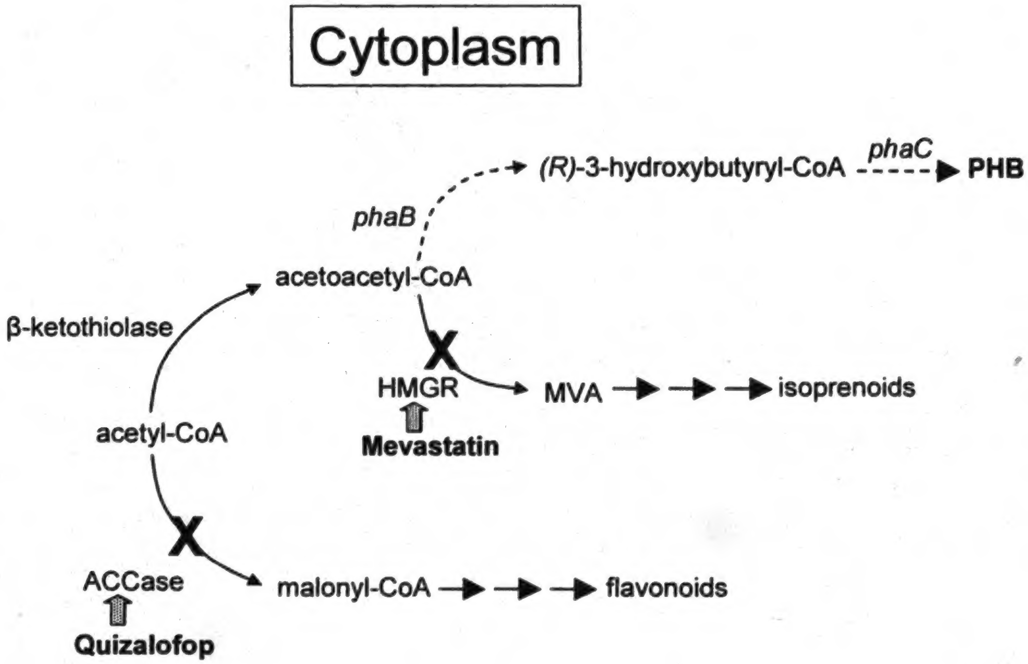

Cytoplasm
(R)-3-hydroxybutyryl-CoA
phaC
PHB
phaB
acetoacetyl-CoA
β-ketothiolase
HMGR
MVA
isoprenoids
acetyl-CoA
Mevastatin
ACCase
malonyl-CoA
flavonoids
Quizalofop

Figure 1-6. Genetically engineered metabolic pathways for PHB synthesis in plant cytoplasm Dashed arrows indicate expression of transgenes, while plant native pathways are shown by solid arrows. Enhancement of acetyl-CoA pool by enzyme inhibitors is also shown. Crosses indicate targeted sites of enzyme inhibitors. Quizalofop inhibits acetyl-CoA carboxylase (ACCase) of the flavonoid synthetic pathway and mevastatin inhibits 3-hydroxy-3-methylglutaryl-CoA (HMGR) of the mevalonic synthetic pathway.

without organelle-specific targeting signals (Figure 1-7a). The reductase and synthase genes were first introduced individually in separate transformations with *Arabidopsis* plants. Transgenic plants expressing both genes were obtained by cross-pollination of the transgenic plants carrying the individual genes. In these hybrids, the presence of PHB granules was observed in various tissues including root, leaf, cotyledon and seed. Moreover, PHB granules were found in some subcellular compartments of the cell including the nucleus, vacuoles and cytosol, but not in the chloroplasts and mitochondria. The highest amount of PHB obtained in these plants was only 0.1% dwt of shoot. Biopolymer from transformed plant cells had the size, appearance, structure and thermal properties of the material from natural bacterial producers. Strong growth retardation and reduction in seed production were observed in transgenic lines with high expression of acetoacetyl-CoA reductase (Poirier et al. 1995; Poirier 2001). Co-expression of acetoacetyl-CoA reductase with PHB synthase led to a further reduction in growth compared to plants expressing only the reductase. It was hypothesized that the diversion of cytoplasmic acetyl-CoA and acetoacetyl-CoA away from the endogenous isoprenoid and flavonoid pathways might have depleted the cell of essential metabolites, thus affecting the growth of the transgenic plants. The isoprenoid pathway contributes to the synthesis of essential plant growth hormones, cytokinins, gibberellins and brassinosteroids. Cytoplasmic acetyl-CoA and acetoacetyl-CoA are involved in the synthesis of sterols, essential components of cell membranes. Therefore, it was likely that small changes in these hormones and sterols severely affected plant growth.

Similar experiments were carried out in black Mexican sweet maize (*Zea mays* L.) and in tobacco (*Nicotiana tabacum* L.). All three *pha* genes from *R. eutropha* were individually introduced under the constitutive CaMV35S promoter. In transgenic cultures, PHB was obtained up to 0.15% of the cell extract dwt. Transgenic cell cultures showed significant growth retardation. Moreover, after weekly sub-culturing over 1.5 years, the activity of PHA synthase gene in the cell cultures was lost. This result suggested that the transgenic lines were genetically unstable (Hahn et al. 1997). The results in transgenic tobacco, carrying *phaB* from *R. eutropha* and *phaC* from *A. caviae* were also similar, with a very low production of PHB occurring at less than 10 µg/g of fresh weight

(Nakashita et al. 1999). Similar results were obtained by expressing *R. eutropha pha* genes in the cytosol of potato and *Brassica napus*. Resultant transgenic exhibited low expression levels for the introduced genes, a low yield of PHB (<0.1% dwt) and stunted growth (Poirier 2002). Interestingly, over-expression of the bacterial ketothiolase in plants expressing the reductase and PHA synthase did not increase PHB yield. Therefore, ketothiolase activity is probably not the limiting factor for PHB synthesis in plants. Instead, other factors including the low flux of acetyl-CoA and differences in codon usage may represent limiting factors.

Cotton fiber is composed of a thin primary wall (0.4 μm) and a thick secondary wall (8-10 μm). The FbL2A promoter is specific to fibers and regulates expression during secondary cell wall formation (Rinehart et al. 1996). Mature cotton fiber contains 89% cellulose. The chemical reactivity, thermal characteristics, water absorption characteristics and the strength of the fiber are all dependent on fiber composition. Attempts have been made to produce PHB in the cotton fiber lumen in order to improve the chemical and thermal properties of the fibers (John and Keller 1996). The *phaB* and *phaC* genes from *R. eutropha* were placed under fiber tissue specific promoters, E6 and FbL2A, and were introduced into cotton genome by particle bombardment. As previously mentioned β-ketothiolase, encoded by the *phaA*, is ubiquitous in the plant cytosol and therefore is not needed to be introduced from *R. eutropha* to produce PHB in plant cell cytosol. These two promoters initiate expression at different stages. E6 has a high activity during early fiber development whereas FbL2A has a higher activity in late fiber development. PHB granules were found in the cytosol of transgenic plants. The level of PHB in transgenic fibers increased during early stages (up to 15 days post anthesis), but due to the large increase in fiber mass that came with plant development, the PHB level eventually decreased. Transgenic plants showed normal growth and morphology. Fibers from transgenic plants contained 0.34% PHB, which was sufficient to improve the insulating properties of the fiber. The transgenic fibers had higher heat capacity and lower thermal conductivity (John 1997). The heat uptake of the fiber might have been influenced by interactions of PHB in the fiber lumen (Chowdury and John 1998).

1.6.2 PHA synthesis in plastids

Low levels of PHB accumulation in the cytosol (<0.5% dwt) have been associated with a limited supply of acetyl-CoA. In contrast, the plastid does exhibit relatively high levels of acetyl-CoA because this organelle is the site of fatty acids biosynthesis, which relies heavily on an acetyl-CoA input. Plastids can accumulate a relatively large amount of starch granules without organelle disruption and therefore should be able to accumulate substantial quantities of PHB. The absence of an endogenous ketothiolase in plastids, however, would necessitate the introduction of bacterial genes encoding ketothiolase along with acetoacetyl-CoA reductase and PHB synthase.

To increase PHB production, expression of the three bacterial genes for PHB synthesis were targeted to plastids using gene constructs that included a DNA segment encoding a chloroplast transit peptide (Nawrath et al. 1994) (Figure 1-7b). The genes were integrated into the nuclear genome, while the recombinant enzymes were translocated into plastids via the chloroplast transit peptide (CTP) of the small subunit of ribulose-bisphosphate carboxylase from pea (Zhong et al. 2003). PHA proteins encoded by the transgenes were synthesized in the cytoplasm, and then post-translationally imported through the chloroplast membrane by binding with a proteinaceous receptor. Afterward a stromal protease catalyzed the cleavage of the transit peptide and left the mature protein free to be functional in the plastids. The transgenes were placed independently under the control of constitutively expressed CaMV35S promoter and were introduced into *Arabidopsis*. Plants with all three enzymes activities (obtained by cross-pollinations of plants expressing individual genes) showed high accumulation of PHB granules (Nawrath et al. 1994). PHB inclusions were observed exclusively in plastids, and the size and appearance of the granules were similar to granules produced in bacteria. The quantity of PHB accumulation increased gradually over time. The maximum amount of PHB in pre-senescing leaves was 10 mg/g fresh weight, which was approximately 14% of the dwt. Plastids of these leaves were filled with PHB granules. There were no major deleterious effects on either plant growth or fertility, although leaf chlorosis was observed in plants accumulating more than 3% PHB. Transgenic plants expressing only PhaB and PhaC were not able to produce PHB, which confirmed the

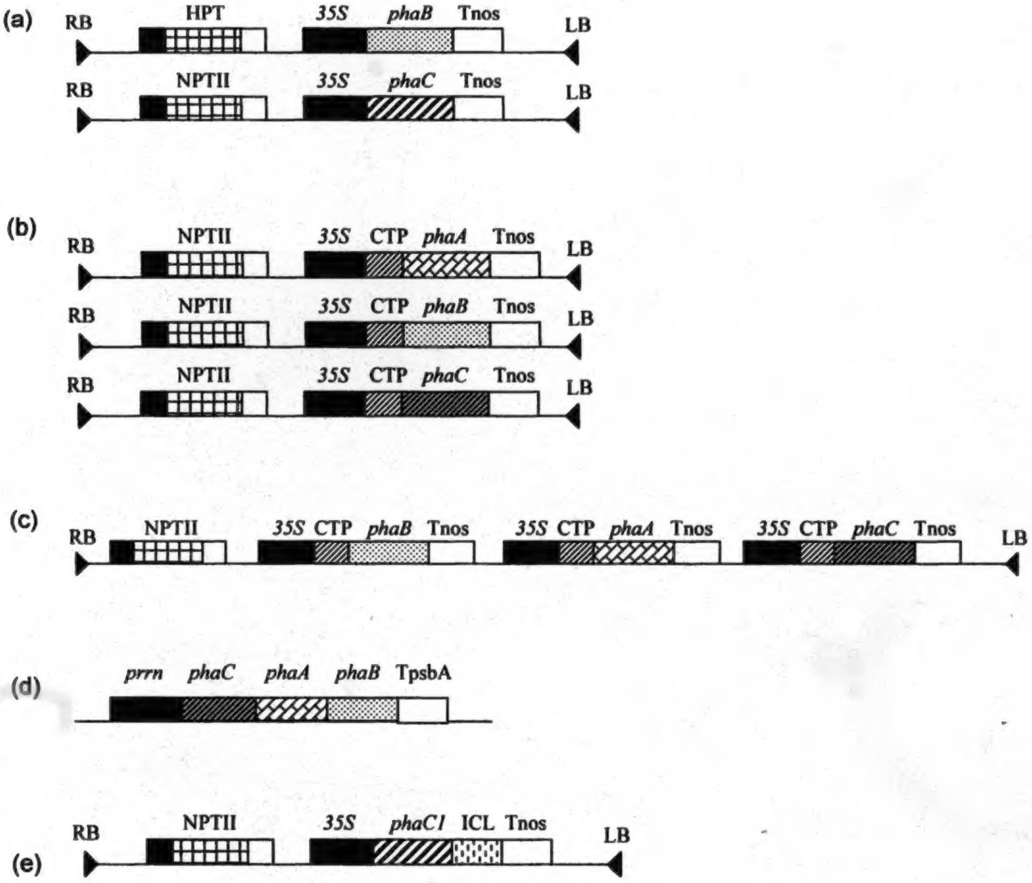

(a)
RB
HPT
35S
phaB
Tnos
LB
RB
NPTII
35S
phaC
Tnos
LB
(b)
RB
NPTII
35S
CTP
phaA
Tnos
LB
RB
NPTII
35S
CTP
phaB
Tnos
LB
RB
NPTII
35S
CTP
phaC
Tnos
LB
(c)
RB
NPTII
35S
CTP
phaB
Tnos
35S
CTP
phaA
Tnos
35S
CTP
phaC
Tnos
LB
(d)
prrn
phaC
phaA
phaB
TpsbA
(e)
RB
NPTII
35S
phaC1
ICL
Tnos
LB

Figure 1-7. Various plant transformation gene constructs:
a) constructs of *phaB* and *phaC* from *R. eutropha* under constitutive CaMV35S (*35S*) promoters with Hygromycin (HPT) and Kanamycin (NPTII) resistance; b) three individual plastid target constructs with each gene attached to the chloroplast transit peptide (CTP) and under CaMV35S promoter; c) triple plastid target construct with CaMV35S promoter and chloroplast transit peptide; d) polycistronic mRNA *pha* operon from *R. eutropha* for chloroplast genomic transformation under *prrn* promoter; e) peroxisomal target construct of *phaC1* from *P. aeruginosa* for mcl-PHA formation with the attachment of DNA segment encoding the last 34 amino acids of *B. napus* isocitrate lysate (ICL). Tnos, terminator sequence from nopaline synthase gene; TpsbA, terminator sequence from plastid *psbA* gene; RB, right border of the T-DNA; LB, left border of the T-DNA

absence of ketothiolase in plastids. Although the production of PHB in these plants was about 100-fold greater than in transgenic lines producing cytosolic PHB, the yield of biopolymer was still lower than the level of endogenous starch accumulation (12 mg/g of the fresh weight). Since the three genes were individually inserted into multiple loci, they did not strictly co-segregate together. This resulted in a decrease in gene copy and PHB production in later generations.

A triple gene construct carrying all three genes in one vector for plastidial PHB synthesis was developed in order to overcome the problems associated with using the crossing approach (Figure 1-7c). Each of the genes in this construct was fused to a DNA segment encoding a chloroplast transit peptide, a CaMV35S promoter and transcription terminator. Transformation into *Arabidopsis* resulted in a dramatic increase in PHB production, up to 40% of the dwt (Bohmert et al. 2000). This amount of PHB was 4-fold higher than the amount produced by plants obtained after crossing those transformed with individual genes. All plastids of mesophyll cells were packed with PHB inclusions in transgenic lines. Plants accumulating a large amount of PHB (30-40% dwt), however, displayed altered morphology, including dwarfism with no seed production. Valentin et al. (1999) performed comparative experiments in this area, and found that transgenic *Arabidopsis* lines carrying three genes in individual constructs produced PHB up to 1.7% of the dwt, whereas lines carrying all of the genes in one construct produced more PHB, up to 13% of the dwt.

Transformation with the triple construct was also applied to alfalfa (*Medicago sativa* L.), an important feed crop. Alfalfa exhibits high biomass production and nutrition, and can adapt to a wide range of environmental conditions. PHB production in alfalfa would improve the value of this crop. After harvest, PHB can be extracted from the leaves, and the leaf byproduct can be used for processing as feed. When the plastidial-targeted triple construct was introduced into alfalfa, transgenic lines produced PHB up to 1.8 mg/g (0.18%) of the dwt. Although the construct used in this transformation was the same as the one used in *Arabidopsis*, transgenic alfalfa produced PHB in a relatively low amount. The investigators hypothesized that this might have been due to gene silencing or the instability of mRNA encoding *pha* genes in alfalfa (Saruul et al. 2002). This plastidial-targeted triple gene construct was further modified to include the HSP70 intron so as to enhance expression in monocots and was introduced into corn. PHB accumulation occurred in the leaves and stalk up to 5.7% dwt (Poirier and Gruys 2001). As in *Arabidopsis*, PHB accumulation gradually increased with time, with older leaves having more biopolymer than younger ones. The granules were unequally distributed, with more granules in bundle sheath cells associated with the vascular tissue than in leaf mesophyll cells. This suggested that more plastidial acetyl-CoA was available for PHB synthesis in certain cells. As in *Arabidopsis*, leaf chlorosis was observed in corn plants accumulating higher amounts of PHB.

Plastids possess their own genomes and can express polycystronic mRNA from an operon. Thus, transformation of plastidial genomes can prevent the outspread of transgenes into wild type plants in the environment. Transformation of tobacco plastids with the *pha* operon (*phaCAB*) of *R. eutropha* under the control of the plastidial rRNA operon promoter (prrn) was performed by gold-particle bombardment (Figure 1-7d). Extract from leaves of the transgenic lines contained only a detectable amount of PHB (Nakashita et al. 2001). Another *pha* operon construct was made using psbA promoter, which is known to have a high expression in plastids. The gene construct was introduced into tobacco (Arai et al. 2004). Transgenic lines produced PHB up to 1.7% of the dwt, but displayed growth retardation and sterility. Position effect and possible mutation of the operon were suggested as reasons for producing low amounts of PHB using plastid operon construct (Lössl et al. 2003). In order to induce PHB synthesis, a nuclear-located, ethanol inducible T7RNA polymerase was constructed as a trans-activation system. T7RNA polymerase was targeted to plastid harboring *pha* operon under T7 regulatory control elements. The treatment of 5% ethanol moderately induced PHB synthesis by 2 folds (Lössl et al. 2005).

Although the high level of PHB accumulation obtained in plastids using nuclear triple gene construct (Bohmert et al. 2000) was encouraging, the poor physical properties of PHB and its limited commercial application were a concern. It was necessary to synthesize copolymers of PHA in plants with better physical properties. As mentioned earlier, copolymers with longer side chains, such as P(HB-HV) have superior properties to PHB. They have lower crystallinity, are more flexible and less brittle. Furthermore, synthesis of PHB and P(HB-HV) rely on production of acetyl-CoA and propionyl-CoA, respectively (Figure 1-3). The production of copolymer in transgenic plants requires expression of 4 transgenes, *ilvA*, *bktB*, *phaB* and *phaC*, as well as the endogenous plastidial pyruvate dehydrogenase complex (PDC) (Figure 1-8), which catalyzes the conversion of 2-ketobutarate to propionyl-CoA. 2-ketobutyrate is also an intermediate in the synthesis of isoleucine. Therefore, PDC would have to compete for 2-ketobutyrate with acetolactate synthase, which is involved in isoleucine biosynthesis (Figure 1-8) from 2-ketobutyrate. To ease this pressure, the quantity of 2-ketobutyrate in plastids was enhanced through the expression of *ilvA*, a threonine deaminase from *E. coli*. The gene product of *phaA*, however, cannot efficiently catalyze the synthesis of 3-ketovaleryl-CoA using propionyl-CoA. Therefore, in order to produce copolymer containing both HB and HV, *BktB*, (from *R. eutropha*) was used as a substitute. This gene encodes a novel β-thiolase having high affinity for both acetyl-CoA and propionyl-CoA. The *E. coli ilvA*, the *R. eutropha phaB*, *phaC* and *bktB* genes were all attached to DNA segments encoding plastidial target sequences and driven by a CaMV35S promoter. Transgenic lines of *Arabidopsis* produced P(HB-HV) copolymer up to 1.6% of the dwt with HV unit fraction of 4-17 mol% (Slater et al. 1999; Valentine et al. 1999). This was one of the most complex genetic engineering experiments performed in plants because the expression of four genes, modified for targeting to the plastid, was required. The transformation involved diversion of carbon from two pathways: acetyl-CoA from fatty acid biosynthesis and propionyl-CoA from amino acid biosynthesis.

As indicated previously, intermediates of fatty acid biosynthesis provide HA monomers for the production of PHA copolymer in some bacteria. 3-hydroxyacyl-ACP-CoA transacylase (PhaG) catalyzes the conversion of *(R)*-3-hydroxyacyl-ACP to *(R)*-3-hydroxyacyl-CoA (Figure 1-5). Attempts to produce PHA copolymer in the plastid of *Arabidopsis* by co-expressing *P. aeruginosa* PHA synthase and *P. putida* PhaG did not lead to PHA accumulation (V. Mittendorf, unpublished results). It is not clear why PHA copolymer synthesis using fatty acid biosynthetic intermediates is difficult to achieve in plastids even though this organelle is a suitable site for PHB and P(HB-HV) synthesis.

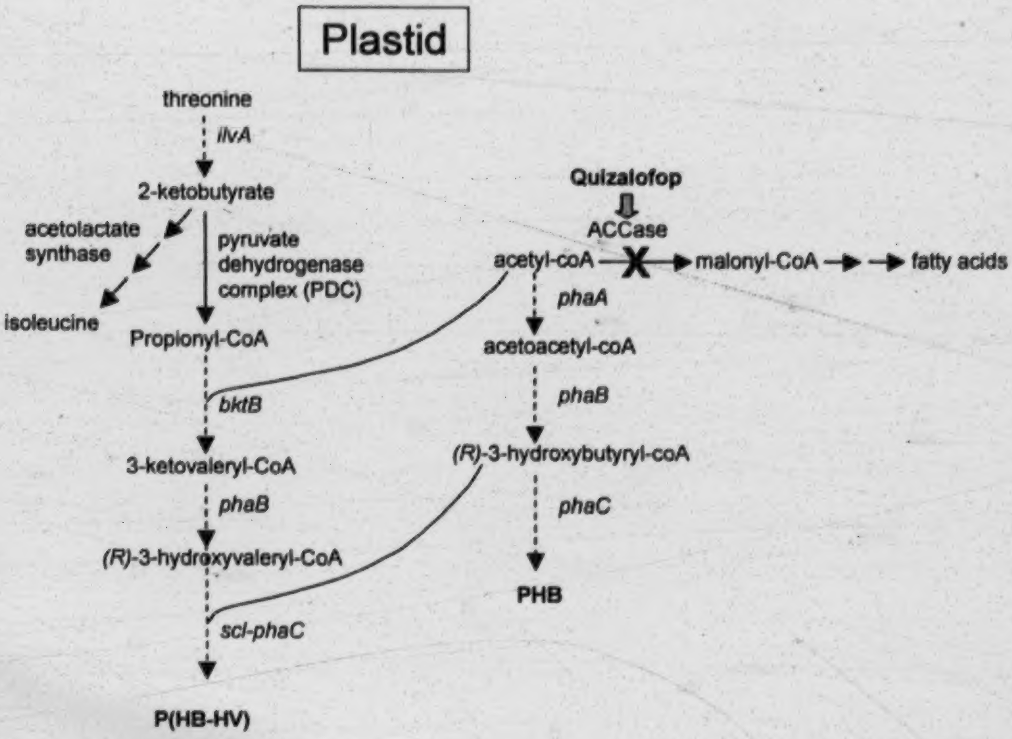

Plastid
threonine
ilvA
2-ketobutyrate
acetolactate synthase
pyruvate dehydrogenase complex (PDC)
isoleucine
Propionyl-CoA
bktB
3-ketovaleryl-CoA
phaB
(R)-3-hydroxyvaleryl-CoA
scl-phaC
P(HB-HV)
Quizalofop
ACCase
acetyl-coA
malonyl-CoA
fatty acids
phaA
acetoacetyl-coA
phaB
(R)-3-hydroxybutyryl-coA
phaC
PHB

Figure 1-8. Genetically engineered PHB and copolymer P(HB-HV) synthetic pathways in plant plastids *ilvA* from *E. coli* encodes threonine deaminase. *phaA, bktB, phaB,* and *phaC* are from *R. eutropha*, and encode β-ketothiolase, 3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase, respectively. PDC refers to pyruvate dehydrogenase complex in plant plastids. The cross indicates the targeted site of enzyme inhibitor, Quizalofop, which enhances the acetyl-CoA pool in the engineered pathway. Dashed arrows indicate expression of transgenes, while plant native pathways are shown by solid arrows.

The high levels of PHB accumulation (up to 40% dwt) achieved by targeting *pha* genes in plastids suggested that plants may serve as suitable factories for the mass production of PHB. The reduction of acetyl-CoA pools in high PHB-producing lines, however, resulted in severe effects on plant growth, fertility and metabolism of sugars when the genes were expressed constitutively in all tissues of the plant. Therefore, it was anticipated that PHA synthesis in plastids of specific tissues, such as seeds, would have smaller adverse effects on plant growth and development. Houmiel et al. (1999) transformed *Brassica napus* with a triple *pha R. eutropha* gene construct. The CaMV35S promoter was replaced with a seed-specific promoter of *Lesquerella fendi* fatty acid hydroxylase. When combined with a DNA segment encoding a chloroplast transit peptide, the expressions of all transgenes were exclusively targeted to leucoplasts, which are enriched in acetyl-CoA for fatty acid synthesis. Transgenic lines carrying the triple construct accumulated PHB up to 8% of the seed dwt (Houmiel et al. 1999; Valentin et al. 1999). PHB production had no deleterious effects on oil production in the seeds, and the size and germination rate of the seeds were normal. The size of the leucoplast, however, was larger in PHB producing seeds, suggesting that leucoplasts can adjust their size to accommodate more granules. This experiment demonstrated that the seed leucoplast was probably a better production system for bioplastic than leaf chloroplast. The leucoplast appeared more metabolically tolerant to the diversion of acetyl-CoA to PHB accumulation compared to leaf chloroplasts. It will be interesting to explore the effects of producing very high levels of this biopolymer in the developing seed.

Synthesis of PHA copolymer was also attempted in the *B. napus* seed leucoplast. The *E. coli ilvA*, and the *R. eutropha phaB*, *phaC* and *bktB* genes were all attached to a plastidial targeting sequence and driven by a seed-specific promoter from the fatty acid hydroxylase gene of *L. fendi*. Transgenic *B. napus* with seed-specific P(HB-HV) construct produced seeds containing up to 2.3% of the dwt copolymer with 6 mol% of HV composition (Slater et al. 1999). There was an inverse relationship between the amounts of PHA to the HV monomer composition. Therefore, it was thought that inefficiency of the PDC in converting 2-ketobutyrate to propionyl-CoA could be a reason for low yield. Further analysis of the transgenic plants revealed that expression of the bacterial *ilvA* resulted in more carbon accumulating in the form of isoleucine or 2-aminobutarate, instead of the target 2-ketobutarate. Nonetheless, synthesis of Biopol™ [P(HB-HV)] in plants was an important milestone towards commercial production of bioplastics in plants.

Accumulation of carbohydrate often takes place in amyloplasts, which are specialized plastids; therefore storage of carbohydrates in these organelles can potentially interfere with PHB accumulation. Sugar beet (*Beta vulgaris* L.) roots, however, store carbohydrates in vacuoles, and not amyloplasts. Therefore, in this root system, PHB accumulation in plastids should not interfere with the accumulation of carbohydrate. Introduction of the three *pha* genes under the CaMV35S promoter with the assistance of the chloroplast transit peptide enabled all the root tissues of sugar beet to accumulate PHB in the amyloplasts of a hairy roots system grown in culture (Menzal et al. 2003). The transgenic plants accumulated PHB up to 3.4% of the dwt. Growth retardation in the transgenic hairy roots occurred in solid and liquid media. The model presented in the hairy root system, however, may not reflect the situation in whole transgenic plant. Nevertheless, this was the first successful demonstration of PHB production in a carbohydrate-storing crop plant.

Plastic is often used to improve the mechanical properties of fiber-based composites. The incorporation of PHAs into fiber-based composites, however, offers a more environmentally friendly alternative. Recently, Wróbel et al. (2004) generated transgenic flax (*Linum usitatissimum* L), which produced bioplastic in the stem. The aim of this work was to improve the mechanical quality of fiber in the plant rather than providing a plant source of PHB for extraction. A triple construct with three *pha* genes of *R. eutropha* was expressed and targeted to plastids in the stem. *phaB* and *phaC* genes were driven by a CaMV35S promoter, whereas *phaA* was controlled by a 14-3-3 promoter. The 14-3-3 promoter expresses specifically in stem tissues. PHB inclusions were found exclusively in the chloroplasts of the stem, and accumulated up to a maximum of 4.62 μg/g of the fresh weight (Wróbel et al. 2004). No growth retardation was observed in the transgenic lines. Interestingly, seed production was enhanced in transgenic lines by a factor of two when compared to non-transgenic control plants.

Furthermore, there were changes in fatty acid composition of the seed oil, which included a decrease in alpha-linolenic acid content. This is the most abundant fatty acid in flax seed oil. The transgenic plants also contained less starch in the chloroplasts and had lower levels of glucose. The presence of PHB in stems improved the elastic properties of the fibers. The Young's modulus E value, a measure of stem tissues resistance to tensile loads, increased up to 2-fold (54.4 Mpa) in transgenic plants. This research demonstrated the feasibility and potential of producing effective biocomposites from stem tissue containing relatively small amounts of PHB.

1.6.3 PHA synthesis in peroxisomes

Peroxisomes are 0.1-1 μm organelles that are bounded by a single lipid bilayer (Brown and Baker 2003). Plant peroxisomes can be categorized as glyoxysomes, leaf peroxisomes and unspecialized peroxisomes. Glyoxysomes are specialized peroxisomes involved in the β-oxidation of fatty acids and directing acetyl-CoA to carbohydrate to generate soluble carbohydrate to support seedling growth (Weselake 2004). Since these organelles produce high levels of acetyl-CoA through β-oxidation, they represent a logical subcellular compartment for production of PHA. In addition, 3-hydroxyacyl-CoA intermediates in the β-oxidation pathway can be used as a precursor in the synthesis of PHAs. Hahn et al. (1999) engineered Black Mexican Sweet maize to produce PHB by introducing three *pha* genes from *R. eutropha*. The construct was designed to target the gene products to peroxisomes. For the targeting process, amino acid residues RAVARL were added to the carboxy terminal of the proteins. PHB accumulated up to 2% dwt in transformants expressing all three enzymes. They have proposed an equilibrium effect to explain this unexpected existence of *(R)*-3-hydroxybytyryl-CoA in plant peroxisomes.

While *R. eutropha* produces PHB, *P. aeruginosa* produces mcl-PHAs. *P. aeruginosa* has a different type of PHA synthase (*phaC1*), which uses 3-hydroxyacyl-CoA intermediates from the β-oxidation pathway as the substrates (Figure 1-9). mcl-PHAs usually have monomers with 2n (n>=0) carbons shorter than the substrates. To make the construct, *phaC1* gene from *P. aeruginosa* was fused to a DNA segment encoding the last 34 amino acids of *B. napus* isocitrate lysate (*ICL*), in order to target the enzyme to leaf peroxisomes and glyoxysomes in cotyledons (Figure 1-7e). The construct was first introduced into *Arabidopsis*. A PTS1 sequence, the terminal amino acids of peroxisomal target sequence, is present in *B. napus ICL*. Expression of the transgene was driven by the constitutive CaMV35S promoter. Transgenic plants grown in light accumulated mcl-PHAs with C6-C16 monomers in peroxisomes, whereas the biopolymer accumulation took place in glyoxysomes when growth occurred in the dark (Mittendorf et al. 1998). The amount of accumulated mcl-PHAs depended on the activity level of the β-oxidation cycle at different stages of development. The activity of the β-oxidation and glyoxylate cycle is high during seed germination and leaf senescence when fatty acids are converted to carbohydrates, and the level of activity drops gradually as the photosynthesis commences in seedlings (Gerhard 1993). The highest level of PHA was detected in 7 days old seedlings with 0.4% of the dwt. PHA level was low in leaves (0.02% dwt), but gradually increased from 2- to 3-fold during leaf senescence. The PHA inclusions were similar in appearance to bacterial inclusions. The PHA inclusions were also observed in vacuoles, which indicated that PHAs could be transported through single-membrane bound organelles (both peroxisomes and vacuoles have single-membranes). No adverse effects on plant growth and seed germination were observed. The peroxisomes were substantially larger in size in transgenic plants and the PHAs accumulated in them were composed of 14 different saturated and unsaturated monomers of 6 to 16 carbons. These monomers could be clearly linked to the corresponding 3-hydroxacyl-CoA generated by the β-oxidation of fatty acids.

PHA synthase utilizes the *R*-isomer of 3-hydroxyacyl-CoA. The process of β-oxidation, however, only generates the *S*-isomer of 3-hydroxacyl-CoA (Graham and Eastmond 2002). The accumulation of a variety of mcl-PHAs in transgenic plants suggested that plant systems contained enzymes that could convert the *S*-isomer into the *R*-isomer. *(R)*-3-hydroxylacyl-CoA epimerase and enoyl-CoA hydratase II present in the plant multifunctional protein (MFP) could potentially be involved in this conversion (Mittendorf et al. 1998, Preisig-Müller et al. 1994) (Figure 1-9). A third route for *R*-isomer production could be hydration of 2-*cis*-enoyl-CoA by the enoyl-CoA hydratase I activity (Schulz 1991). In bacteria, *(R)*-3-hydroxyacyl-CoA is generated by

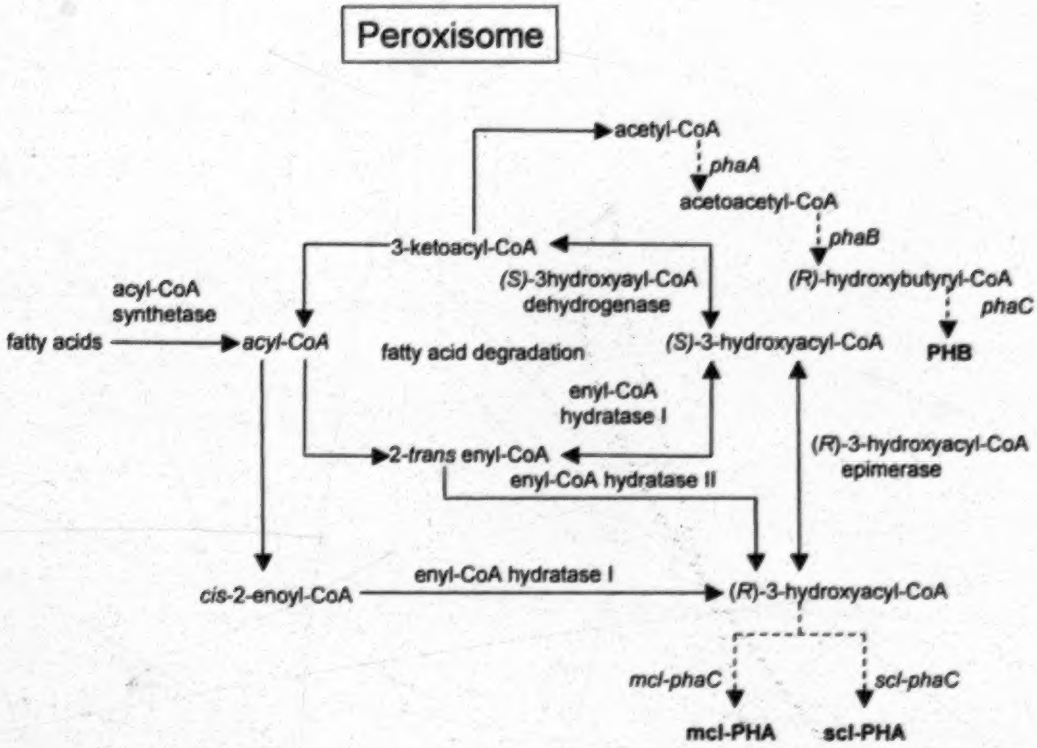

Peroxisome
acetyl-CoA
phaA
acetoacetyl-CoA
phaB
3-ketoacyl-CoA
(S)-3hydroxyayl-CoA
dehydrogenase
(R)-hydroxybutyryl-CoA
phaC
acyl-CoA
synthetase
fatty acids
acyl-CoA
fatty acid degradation
(S)-3-hydroxyacyl-CoA
PHB
enyl-CoA
hydratase I
(R)-3-hydroxyacyl-CoA
epimerase
2-trans enyl-CoA
enyl-CoA hydratase II
enyl-CoA hydratase I
cis-2-enoyl-CoA
(R)-3-hydroxyacyl-CoA
mcl-phaC
scl-phaC
mcl-PHA
scl-PHA

Figure 1-9. Genetically engineered metabolic pathways of scl- and mcl-PHA formation in plant peroxisomes Expressions of transgenes are indicated by dashed arrows. *phaC* from *A. caviae* and *P. aeruginosa* encode PHA synthases that use (*R*)-3-hydroxyacyl-CoA from fatty acid degradation as substrate for scl- and mcl-PHA polymerization, respectively.

a 3-hydroxyacyl-CoA epimerase, an *R*-specific enoyl-CoA hydratase II, or a 3-ketoacyl-CoA reductase (Steinbüchel 1991) (Figure 1-4).However, yeast only contains an enoyl-CoA hydratase II (Hiltunen et al. 1992).

Early attempts at producing mcl-PHAs (C6-C14) resulted in biopolymers that were too elastic. They contained a high proportion of monomers larger than 10 carbons, and a high proportion of unsaturated monomers. Such PHA polymers have a low melting point and behave like glue at room temperature. Increasing the proportion of shorter chain length monomers (e.g., C4-C8) in PHA copolymer can solve this problem. PhaC synthase from *A. caviae*, which produces scl-PHA copolymers with C3-C7 hydroxy fatty acids, was introduced into *Arabidopsis* in an attempt to improve the quality of the resulting biopolymer (Arai et al. 2002). The expression of transgenes was driven by the CaMV35S promoter. The construct contained a DNA segment encoding a 10 amino acid stretch from the carboxy-terminal of spinach glycolate oxidase attached to direct the enzyme to peroxisomes. Transgenic plants produced scl-PHA copolymers P(HB-HV-HH) containing C4-C6 monomers with improved properties. This type of scl-PHA copolymer has better commercial potential because it is more flexible than PHB and less elastomeric than mcl-PHA. scl-PHA accumulated up to 441.7 μg/g (0.044%) of the leaf dwt. There was a wide range of scl-PHA accumulation among different organs and stages of development, suggesting different activity levels of β-oxidation throughout the plant. β-oxidation levels are high during seed germination and reduced after the commencement of photosynthesis. This metabolic difference was reflected in a high level of PHA accumulation (120 μg/g of the dwt) in 7 day-old seedlings and only 2.2 μg/g in the leaves of 28 day-old plants. The level of PHA increased to 230 μg/g in the senescing leaves of 60 day-old plants when β-oxidation increased again.

1.6.4 Modulating the quantity and monomer composition of PHA in transgenic plants

It may be possible to modulate PHA accumulation in the peroxisome by altering the carbon flux to the β-oxidation cycle because PHA synthesis draws upon intermediates of fatty acid degradation. Indeed, production of PHA has been used as a tool to analyze carbon flow through the β-oxidation cycle (Poirier 2002). Plastidial acyl-acyl carrier protein (ACP) hydrolase catalyzes the removal of the growing fatty acid chain from the fatty acid synthase complex (Ohlrogge and Browse 1995). Previous studies with *B. napus* expressing California bay (*Umbellularia california*) lauroyl-ACP hydrolase revealed that developing seeds accumulating lauric acid in triacylglycerol produced a substantial portion of lauric acid that could be recycled through the β-oxidation pathway (Eccleston et al. 1996). These results suggested that expression of an acyl-ACP hydrolase specific to a particular fatty acid might be a way of increasing carbon flux towards β-oxidation and peroxisomal PHA synthesis derived from that particular fatty acid. A transgenic *Arabidopsis* line co-expressing a acyl-ACP hydrolase from *Cuphea lanceolata* along with PHA synthase produced 8-fold more PHA in plant shoots than transgenic lines carrying PHA synthase alone (Mittendorf et al. 1999). The mcl-PHA in the transgenic plant carrying both genes was enriched in saturated 3-hydroxyacid monomers containing 10 carbons or less (40 mol% 3-hydroxydecanoic acid, 32 mol% hydroxyoctanoic acid and 4 mol% hydroxyhexanoic acid). This result suggested that expression of acyl-ACP hydrolase channeled decanoic acids towards peroxisomal β-oxidation (Figure 1-10). Poirier et al. (1999) extended the strategy of synthesizing mcl-PHA enriched with specific polymers in vegetative tissues to developing seeds. In mature seeds of *Arabidopsis*, the investigators obtained up to 0.1% dwt in PHA copolymers. These results demonstrated that manipulation of genes involved in the synthesis of unusual fatty acids could be used to modulate the quantity and monomer composition of mcl-PHAs. The amount of PHA synthesized in peroxisomes (<1% dwt) by co-expressing acyl-ACP hydrolase and PHA synthase was still much lower than the amount of PHB that could be synthesized in plastids (40% dwt). Further studies on mcl-PHA synthesis in *S. cerevisiae* and *P. pastoris* might eventually provide insight into this metabolic limitation on PHA production in plant peroxisomes (Poirier et al. 2001, 2002).

Placing limitations on triacylglycerol accumulation in developing seeds has also been shown to affect the amount of PHA formed in peroxisomes. Poirier et al. (1999) conducted studies on PHA formation using the *tag1* mutant of *Arabidopsis*, which is deficient in diacylglycerol acyltransferase (DGAT) activity. The decreased incorporation

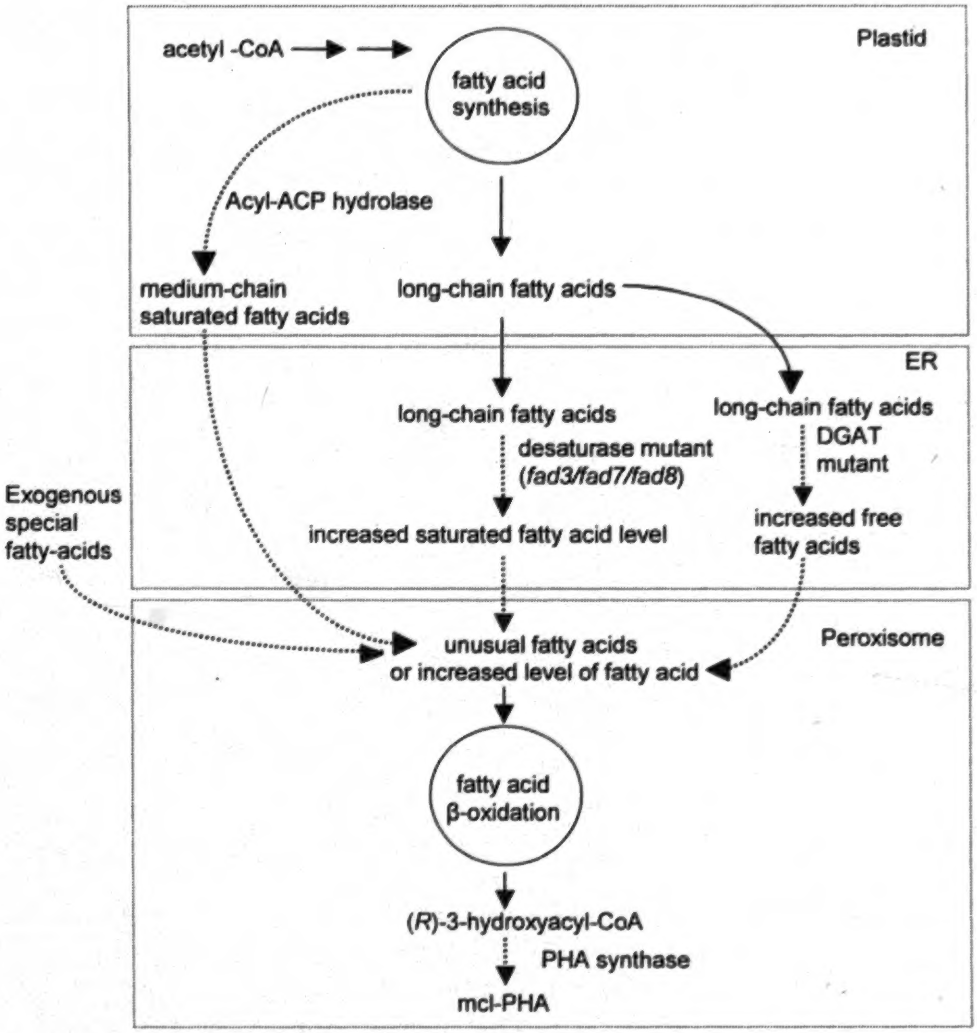

Plastid
acetyl -CoA
fatty acid synthesis
Acyl-ACP hydrolase
medium-chain saturated fatty acids
long-chain fatty acids
ER
long-chain fatty acids
desaturase mutant (*fad3/fad7/fad8*)
increased saturated fatty acid level
long-chain fatty acids
DGAT mutant
increased free fatty acids
Exogenous special fatty-acids
Peroxisome
unusual fatty acids or increased level of fatty acid
fatty acid β-oxidation
(*R*)-3-hydroxyacyl-CoA
PHA synthase
mcl-PHA

Figure 1-10. Strategies of synthesizing PHA in peroxisomes with special monomers, using lipid biosynthesis mutant (e.g. DGAT mutant); co-expressing fatty acid biosynthetic genes (e.g. acyl-ACP hydrolase); exogenous feeding of special fatty acids, etc. (adapted from Poirier 2002).

of fatty acids into triacylglycerol resulted in an increased level of free fatty acids in seeds (Figure 1-10). It was anticipated that excess fatty acids would be channeled towards β-oxidation resulting in an increased availability of intermediates for mcl-PHA synthesis. Indeed, when the peroxisomal-targeted PHA synthase was expressed in this mutant, there was a 10-fold increase in mcl-PHA in the transgenic seeds compared to expression of the PHA synthase in the wild-type plant (Poirier et al. 1999).

Another mutant of *Arabidopsis*, known as *fad3/fad7/fad8*, was engineered to obtain PHA with specific monomers. This triple mutant is deficient in synthesis of tri-unsaturated fatty acids. mcl-PHA produced in this mutant was almost completely deficient in all 3-hydroxyacids derived from the degradation of tri-unsaturated fatty acids (Mittendorf et al. 1999). These results suggested that it is possible to modulate the monomer composition of PHA in engineered plants by altering the degree of unsaturation of fatty acids.

Another approach used to alter the monomer composition of PHA synthesized in transgenic plants involves the exogenous application of specific fatty acids (Figure 1-10). Mittendorf et al. (1999) grew transgenic *Arabidopsis* plants expressing a peroxisomal-targeted *P. aeruginosa* PHA synthase gene in liquid medium supplemented with various fatty acids. This resulted in a significant increase in mcl-PHA containing monomers derived from the β-oxidation of the externally supplied fatty acids. For example, addition of tridecanoic acid (C13:1 Δ12) resulted in the production of PHA containing mainly unsaturated odd-chain monomers. In contrast, adding Tween-20 (polyethylene sorbitan monolaurate) to the growth medium resulted in the incorporation of even-numbered, saturated C6-C12 monomers derived from Tween-20. The investigators reported that Tween-20 induced the activity of MC-acyl-CoA oxidase (ACOX) and LC-ACOX. More recently, Arai et al. (2002) demonstrated that application of Tween-20 also resulted in 4-fold increase in scl-PHA in *Arabidopsis* plants expressing an *A. caviae* PhaC synthase in the peroxisomes. This PHA synthase catalyzes the production of scl-PHA copolymers. The increased synthesis of scl-PHA in these *Arabidopsis* plants was due to incorporation of monomers derived from fatty acid moieties of Tween-20 that have been shunted through β-oxidation cycle. Thus, it was suggested that Tween-20 activated β-oxidation of scl-fatty acids and also probably induced SC-ACOX activity.

Although the yield of PHA in these experiments did not reach a commercially viable level, the results demonstrated that PHA quantity and monomer composition could be altered in the plant peroxisome by supplying specific fatty acid substrates to the β-oxidation cycle. As well, these investigations demonstrated that the plant β-oxidation cycle is capable of generating a large spectrum of monomers from fatty acids that are not naturally present in plants. Therefore, this method can be utilized in the analysis of the degradation of an unknown or unusual fatty acid in plants (Daae and Dunnill 1999; Allenbach and Poirier 2000; Poirier 2002; Moire et al. 2004). Applying exogenous fatty acids is not a cost-effective option for PHA production in plants. The results of experiments with exogenously applied fatty acids, however, can provide us with clues on how we might alter fatty acid production in plant systems to generate PHAs of desired monomer composition.

There appears to be a major metabolic advantage to producing PHA in the peroxisome. Carbon used for PHA synthesis in the peroxisome is not diverted from anabolic pathways involved in the synthesis of essential compounds like fatty acids and amino acids, but instead is derived from catabolic pathways. Therefore, a high level of PHA synthesis in the peroxisome might not have as an adverse effect on plant growth and development as it would in a transgenic plant system that generated PHAs from precursors produced through anabolic pathways such as fatty acid synthesis in the plastid.

1.6.5 Barriers to increasing PHA production in plants

Production of PHA in transgenic plants has barriers associated with expression of transgenes and metabolic load on plant growth. The constitutive expression of one of the PHA synthesis genes (*phaA*) is considered a crucial obstacle (Poirier et al. 2000; Bohmert et al. 2002). Bohmert et al (2002) demonstrated that constitutive expression of *phaA* was detrimental to plant growth as early as during the transformation steps. Expression of this gene was responsible for drastic reduction of transformation efficiency in potato and tobacco. It was suggested that the toxic effects of *phaA* could result from PHB biosynthesis intermediates, the depletion of acetyl-CoA pool, or unexpected interactions between β-ketothiolase with other substrates in the plastids. Preventing the expression of *phaA* during transformation/regeneration procedure by using inducible promoter allowed the generation of transformants. Use of inducible promoter also resulted in two fold increase in PHB production without alteration in phenotype, compared to *Arabidopsis* lines constitutively expressing the *phaA* gene (Bohmert et al. 2000). However, use of inducible promoter in potato and tobacco, although resulted in generation of transformants, the amount of PHB formed was nevertheless rather low (<0.1% dwt). This suggested that what was true for *Arabidopsis* might not necessarily true for other plant species.

Several approaches were undertaken to improve the yield of PHB in plants. Acetyl-CoA is not only a substrate in PHB synthesis, but this thioester is a crucial substrate in several metabolic pathways including flavonoid and isoprenoid synthesis in cytoplasm, and fatty acid synthesis in plastids (Figure 1-6, 1-8). Specific enzyme inhibitors were used to suppress these anabolic pathways in order to increase the availability of acetyl-CoA for PHB production. Quizalofop (an herbicide) inhibits acetyl-CoA carboxylase, which converts acetyl-CoA to malonyl-CoA. Application of Quizalofop was shown to increase PHB production in the cytosol and plastid by 170% and 150%, respectively. Mevastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMGR), which has a roll in the mevalonic acid synthetic pathway in cytosol (Figure 1-6). Application of 1 μM of mevastatin increased PHB production by 192% (Suzuki et al. 2002), and interestingly, no effect on plant growth was observed. This suggests increasing the availability of acetyl-CoA in plant cell might be a strategy to improve PHB yield.

1.7 PHA extraction

The extraction of bioplastic from biomass poses yet another challenge. There are two common protocols used for PHA extraction from bacteria. The conventional one is based on the solubility of PHA in chloroform and insolubility in methanol (Kessler et al. 2001). After harvest, lipids and other lipophilic components in the bacterial cells are removed by reflux in hot methanol followed by solubilization of PHA in warm chloroform. PHA from the chloroform solvent can be recovered by solvent evaporation or precipitation by addition of methanol. Although highly purified PHA is obtained by this method, a large amount of hazardous solvent is needed. Thus, this method is not environmentally friendly and unsuitable for mass production of bioplastic (Byrom 1987). The second protocol is designed to avoid the use of organic solvents. Bacterial cells are treated with a cocktail of enzymes (including proteases, nucleases and lysozymes) and detergents to remove proteins, nucleic acids, and cell walls, leaving the PHA intact (Byrom 1987).

In the large scale production of PHA in crops, the extraction and purification of PHA from biomass is a critical factor for determining the practical feasibility of the technology. It is important that PHAs from transgenic plants can be extracted efficiently and easily, much like the extraction of endogenous compounds, such as starch, sucrose and oil. Unlike extractions of bacteria, which are specifically intended for PHA production, there are other useful byproducts that can also be extracted from harvested crops. Any extraction process from plant tissue should accommodate extraction of such compounds in unmodified form. The conventional methods used for extraction of low molecular weight lipids are not applicable for bioplastic produced in plant cells. Unlike separating vegetable oils from oilseeds, PHAs cannot be squeezed from the seeds by applying mechanical pressure. Solvent extraction is also difficult because the resulting polymer solution is extremely viscous, making the solution very difficult to work with. Also, the removal of solvent from the polymer is a slow and difficult process, and separations based on sedimentation are extremely slow.

On a laboratory scale, the extraction of PHA from plant tissue has usually relied on the same method used to extract the polymer from bacteria (i.e., chloroform and methanol). Components of the recovered PHA are then analyzed using various analytical methods such as gas chromatography/mass spectrometry. There are no publications available in scientific literature regarding large scale PHA extraction from plant tissues. There are methods, however, based on both solvent and non-solvent procedures issued in the form of patents (Poirier 2001).

In oilseeds, the oil was recovered separately from PHA, and the residual seed meal was used as animal feed (Noda 1997, 1998a). The seeds containing both oil and

PHA were crushed, and the oil was obtained by a combination of pressing and extraction with a solvent that did not dissolve PHA (e.g., hexane). The defatted compounds containing PHA were then extracted with a solvent that solubilized PHA, leaving the meal byproduct behind that was rich in protein. By increasing the temperature and pressure, and with the right choice of PHA copolymer, a range of less hazardous 'PHA-poor' solvents was used to extract PHA. For example, P(HB-HV) became soluble in methanol when the temperature was raised to 120°C. After solubilization, PHA was recovered by cooling, adding non-solvent and evaporating the solvent (Martin et al. 1997; Noda 1997, 1998a; Kurdikar et al. 1998, 2000). PHA was also chemically transformed into a PHA derivative and separated from the mixture using distillation, extraction or chromatography (Martin et al. 2004). The solvent extraction method was further improved by the use of marginal non-solvents, such as alkanes, alcohols or even oil (Noda 1998a). Marginal non-solvents (e.g., acetone) do not effectively dissolve PHA by themselves, but become competent when mixed with a PHA solvent. Both oil and PHA were extracted from the seeds by crushing in acetone. After the removal of acetone, oil, which alone cannot dissolve PHA polymers, became an effective suspending medium for the precipitating PHA. Horowitz and Brennan (2002) recommended the use of ozone in PHA-containing slurries and suspensions, either in water or in solvent, to obtain odor-free PHA having an enhanced level of purity.

Similar to non-solvent PHA extraction from bacteria, the use of a cocktail of enzymes has also been suggested for extraction of PHA from plant tissues (Liddell 1997). Oil in the crushed seeds containing PHA was first recovered by hexane. The defatted meal was then treated with enzymes, surfactants, oxidizing agents and detergents to digest the non-PHA components such as carbohydrates, proteins and nucleic acids. The PHA granules were recovered by decantation, filtration and centrifugation. It is not clear, however, if this protocol would be realistic and cost effective in an industrial process.

Two new methods for PHA extraction have been described which were based on wet and dry milling methods used in the corn industry. One method is based on air classification, which separates dry solid components according to weight and/or size (Noda 1998b). The other method is based on centrifugal fractionation, which separates particles in solution based on size and/or density (Noda 1999). Because PHA granules are the smallest particles (0.2-1 μm) present in plant cells, compared to starch grains or protein bodies, this size difference can be used to separate them from the other plant components.

1.8 Conclusions

Research into the production of PHAs as petrochemical alternatives for the future has been explored using bacterial and plant systems. Great advances have been made in the protein engineering of PHA synthases to alter their specificity properties so as to produce PHAs with desired monomer composition. Bacterial fermentation, however, relies on external carbon sources such as glucose. Synthesis of PHA in plants, which relies on carbon dioxide and light, represents a more cost-effective approach to produce this biopolymer in large quantities. In plant systems, several ingenious approaches have been used to capture intermediates of carbon catabolism and convert them into PHA. Different compartments of the plant cell and different tissues of the plant have been examined for their suitability in producing and storing PHA granules. A major challenge in producing commercially viable levels (> 15% dwt) of PHA in plant tissue is being able to do this without compromising the normal growth and development of the plant. Production of PHA in agricultural crops is likely to be economically viable if it can be produced as a byproduct with some other plant constituent such as oil or starch. For example, if PHA is produced in an oilseed crop, the bioplastic can potentially be recovered along with the oil fraction leaving the remaining meal for use as animal feed. At this point, levels of up to 8% dwt of the seed are possible without deleterious effects on plant growth (Houmiel et al. 1999). Improving the yield of PHA in plants along with a desired monomer composition represents an ongoing challenge (Snell and Peoples 2002). More efficient methods need to be developed for multiple-gene transformation. Plastid transformation might be an alternative for multiple-gene transformation because the plastidial genomes are maternally inherited. Although previous attempts at plastidial transformation resulted in very low accumulation of PHB, new methods of high-level expression designed for this organelle (De Cosa et al. 2001) hold promise. Since high levels of PHA disrupt chloroplast function, it might be worthwhile attempting to increase the number of chloroplasts per cell to obtain more PHA per g tissue.

The cost of mass production of PHAs in bacteria is prohibitive except for certain specialty bioplastics used in medical applications. The synthesis of PHA in bacteria and subsequent extraction of the biopolymer is estimated to cost approximately 3-4 US$/kg (Lee et al. 1997). This is 5-10 times more expensive than petroleum based polymers, such as polypropylene (<1$/kg). In this context, the synthesis of PHA in crop plants can be regarded as a promising alternative for the large-scale and low cost production of this polymer. Producing PHA in plants, however, will likely be more expensive than producing corn starch or soybean oil, which cost in the range of 0.25-0.50 US$/kg. Expenses associated with PHA extraction from plant sources will represent a relatively large component of the total cost of producing this polymer in the plant. Although a few methods have been developed for large-scale extraction of recombinant proteins from plants (Menkhaus et al. 2004), the extraction and processing of PHA from plant tissue still requires considerable research.

1.9 References

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Chapter 2 Development of *phb* gene constructs

2.1 Introduction

2.1.1 PHB synthesis in a natural producer, *Ralstronia eutropha*

Ralstronia eutropha, the most well-known and extensively studied PHB producing bacteria, has the potential to generate the highest yield of PHB among the natural producers discovered so far (Madison and Huisman 1999). *R. eutropha* utilizes glucose or other types of sugar as a carbon source to generate acetyl-CoA, which is the primary substrate for PHB synthesis. The PHB synthesis pathway is comprised of three enzymatic reactions; the first reaction is a condensation of two acetyl-CoA molecules by β-ketothiolase, which gives one acetoacetyl-CoA molecule as a product (Figure 2-1). Then acetoacetyl-CoA gets reduced by acetoacetyl-CoA reductase to become a 3-hydroxybutyryl-CoA (HB) monomer. The HB monomers are combined together through a polymerization reaction by PHB synthase (Madison and Huisman 1999). Three genes that are responsible for the three step reactions are *phbA*, *phbB*, and *phbC*. In *R. eutropha*, these three genes are located in the same operon, *phbCAB*, with sizes of *phbA* 1.2 kb, *phbB* 0.7 kb, and *phbC* 1.8 kb (Figure 2-1).

2.1.2 Engineering PHB synthesis pathway in cytosolic and plastidial compartments

Cytosol was targeted as the first site for PHB synthesis because the bacterial enzymes could be directly expressed in cytoplasm without any modification of the proteins. Furthermore β-ketothiolase, the first critical enzyme for PHB synthesis, is ubiquitously found in higher plant cytoplasm as a part of isoprenoid production. Thus, in order to introduce PHB synthesis pathway into cytosol, expression of only two transgenes from *R. eutropha* (*phbB* and *phbC*) is required (Moire et al. 2003) (Figure 2-2a).

The plastid is the main compartment of the cell in which fatty acid synthesis takes place. Plastids naturally have a much larger acetyl-CoA pool than cytoplasm. Therefore by diverting acetyl-CoA in plastids to PHB synthesis pathway, higher yield of PHB is expected to be produced than in the cytoplasm. However unlike in cytoplasm, there is no expression of β-ketothiolase found in the plastids. In this case, all of the three *phb* genes have to be introduced, as well as a target signal attached to each of them, to direct the expressions of the enzymes into the plastids. The target signal is also referred to as chloroplast transit peptide sequence (CTP) (Nawrath et al. 1994) (Figure 2-2b).

2.1.3 Seed-specific Napin promoter and chloroplast transit peptide signal

Napin protein is one of the main seed storage proteins found abundantly in oilseed rape (*Brassica napus*) and related species, and is encoded by multigenic family with 10-16 members (Scarafoni et al. 2001). During embryogenesis in flowering plants, Napin is synthesized and accumulated in the cotyledon and axis cells of the embryo during seed maturation. Upon germination, Napin and other storage proteins are subsequently degraded, providing a source of nitrogen and sulfur for the early growth of the seedlings. The *napin* genes are exclusively expressed in immature or developing seeds (Stayton et al. 1991). Thus, the promoter region of this gene (1.1 kb) has been isolated and become one of the most widely used seed-specific promoters to direct expression of a transgene to developing seeds.

Rubisco is a bi-functional enzyme abundantly found in chloroplasts of plant cells. It catalyzes the first step of Calvin cycle and photorespiration. Rubisco consists of eight of each large and small subunits, LSU and SSU respectively. LSU is encoded by chloroplast genome where the photosynthesis takes place, while SSU is encoded by the nuclear genome. The precursors of SSU are formed in the nucleus; it contains an extension of terminal amino acids called a chloroplast transit peptide (CTP). This CTP has an important role in transportation of the SSU into the chloroplast (Ellis 1981). The CTP sequence was isolated and utilized in targeting a protein expression into the chloroplast. It has been reported that an additional first 24 amino acids of the mature SSU are required for targeting some proteins into the chloroplasts (Corbin et al. 2001, Nawrath et al. 1994). In this study CTP along with the first 24 amino acids of SSU mature protein of *A. thaliana* was fused in-frame with a gene, to facilitate the transportation of the target protein into chloroplast. By combining together the use of seed-specific Napin promoter and CTP sequence, the expression of transgenes in gene constructs developed in this study were targeted specifically to leucoplasts, which are a type of plastids in developing seeds.

2.1.4 Summary

In this study three genes, which encode three enzymes essential for PHB synthesis, were isolated from a natural producer *R. eutropha* by colony PCR technique. Plastid target signal fragment (chloroplast transit peptide sequence, CTP), was isolated similarly by PCR using *A. thaliana* genomic DNA template. *phb* genes and CTP fragment were sub-cloned into cloning vectors, and sequenced to confirm no mismatch in the nucleotide sequences. Three gene constructs were prepared by series of restriction and ligation reactions of the genes to appropriate promoters, selectable markers and target signals. The first gene construct is a *phbB* and *phbC* double-cassette construct with each driven by a seed-specific Napin promoter. This gene construct was used to introduce PHB synthesis pathway into cytosolic compartments of developing seeds. The other two gene constructs were designed to carry different selectable markers for co-insertion into the same plant line. The one with Kanamycin resistant marker was a *phbA* and *phbB* double-cassette construct with each driven by a seed-specific promoter and attached to a CTP sequence. The other with PPT herbicide resistant marker, was a *phbC* single gene construct, which also was under a seed specific promoter and attached to a CTP sequence.

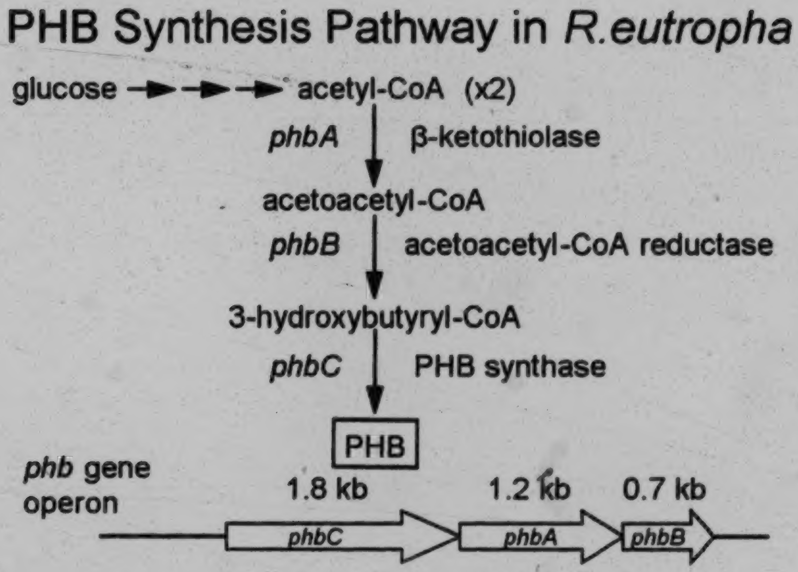
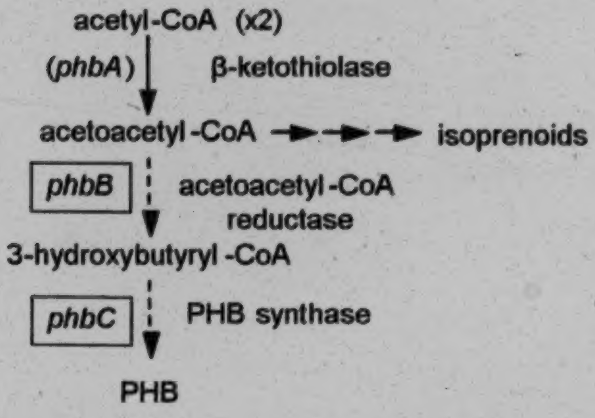

PHB Synthesis Pathway in R.eutropha
glucose
acetyl-CoA (x2)
phbA
β-ketothiolase
acetoacetyl-CoA
phbB
acetoacetyl-CoA reductase
3-hydroxybutyryl-CoA
phbC
PHB synthase
PHB
phb gene operon
1.8 kb
1.2 kb
0.7 kb
phbC
phbA
phbB

Figure 2-1. PHB biosynthetic pathways in *R. eutropha*

phbA encodes β-ketothiolase, enzyme involved in condensation of two acetyl-CoA molecules. Acetoacetyl-CoA reductase (encoded by *phbB*) reduces acetoacetyl-CoA to form a 3-hydroxybutyryl-CoA monomer. *phbC* encodes PHB synthase, the last enzyme responsible for polymerization of the monomers. In *R. eutropha*, the three *phb* genes are located in the same operon with sizes of *phbA* 1.2 kb, *phbB* 0.7 kb, and *phbC* 1.8 kb.

a. Engineered PHB Synthesis in Cytosol


acetyl-CoA (x2)
(phbA)
β-ketothiolase
acetoacetyl-CoA
isoprenoids
phbB
acetoacetyl-CoA reductase
3-hydroxybutyryl-CoA
phbC
PHB synthase
PHB

b. Engineered PHB Synthesis in Plastid

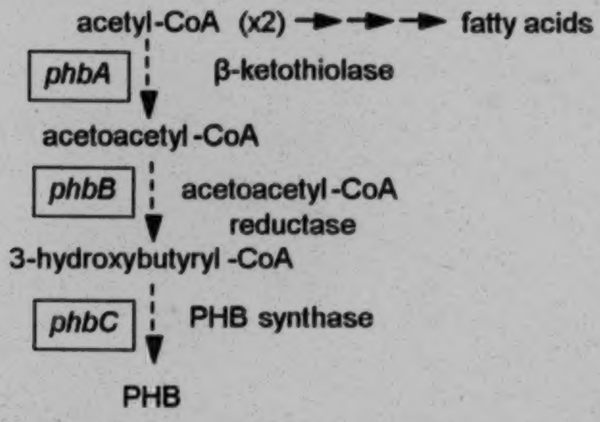

acetyl-CoA (x2)
fatty acids
phbA
β-ketothiolase
acetoacetyl-CoA
phbB
acetoacetyl-CoA reductase
3-hydroxybutyryl-CoA
phbC
PHB synthase
PHB

Figure 2-2. Engineering PHB synthesis in cytosol and plastid

Dashed arrows indicate expression of transgenes, while plant native pathways are shown in solid arrows. a.) in cytosol, expression of β-ketothiolase is naturally found in isoprenoids synthesis pathway. Transgenes required to be introduced are *phbB* and *phbC*, shown in boxes. b.) in plastids, introduction of all three *phb* genes are required.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

R. eutropha strain H16, ATCC number 17699, was used for isolation of *phb* genes. The freeze-dried stock was purchased from American Type Culture Collection (ATCC), and grown on tryptone soy agar (TSA) plates or cultured in tryptone soy broth (TSB) at 28°C under an aerobic condition (Peoples et al. 1989a). Culture of *R. eutropha* for long term storage was prepared by TSB culture after shaking for two nights at 28°C with additional glycerol to a final concentration of 10% (v/v) and stored at -80°C.

Escherichia coli strain DH5α was used throughout the process of gene construct preparations. *E. coli* was grown on Luria-Bertani (LB) plates or cultured in LB broth at 37°C, supplemented with appropriate antibiotics. Long-term storage of liquid cultures were prepared after shaking overnight with additional glycerol to a final concentration of 10% (v/v) and stored at -80°C.

2.2.2 Plant material and growth condition

Arabidopsis thaliana ecotype Wassilewskija (WS) seeds were sown on surface of moistened soil (SUN GRO Mix1, Sun Gro Horticulture Inc., Vancouver, BC) in 6 inch pots. The pots were covered with saran wrap forming a dome shape to prevent the soil from drying, and placed in a growth chamber (24 hours photoperiod, 22°C 16 hours/ 20°C 8 hours cycle) for 1 week. After germination, the saran wrap was gradually removed to let the plants slowly acclimatize to conditions in the growth chamber. The plants were kept in the growth chambers, watered as required until approaching stage of use.

2.2.3 Vector descriptions

Names, characteristics and selectable markers of vectors used in this study are listed in table 2-1. pBluescript SKII(-) and pGEM-T cloning vectors were used in gene isolations and sequence confirmations. Modified pGEM-T vector, pNAP-rbc-GEM-T, plant gene expression vector pKYLX-NAP with seed-specific Napin promoter, and pRD320 carrying *pat* gene for herbicide resistance fragment were used to prepare three seed-specific gene constructs.

2.2.4 *A. thaliana* genomic DNA extraction from leaf tissue

Young fresh leaf material was collected from 2 week-old plants, and immediately placed in liquid nitrogen. 100 mg of the frozen tissue was ground with autoclaved mortar and pestle till fine powder was obtained. Genomic DNA was extracted and purified using the Mini DNeasy Plant Kit (Qiagen Inc., Mississauga, ON). The ground tissue samples were lysed in the lysis buffer provided at 65°C for 10 minutes. RNase was added to the lysate to degrade contaminated RNA. Precipitates and cell debris were separated by filtration through a spin column by centrifugation. Proteins and polysaccharides were removed by adjustment of buffering conditions with solutions provided. Genomic DNA in the lysate was captured onto the silica gel membrane in another spin column, while other impurities passed through by centrifugation. The DNA was washed twice with wash buffer to remove remaining contaminants. Pure genomic DNA was eluted in sterile distilled water, and stored at -20°C.

2.2.5 PCR amplification and gel electrophoresis

PCR reaction was performed with a mixture of following components: 5 μL 10xPCR buffer, 1 μL dNTP mix (25 mM), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 0.5-3.5 μL $MgCl_2$ (50 mM) according to primers and polymerases used, DNA template (bacterial colony, plasmid DNA, or genomic DNA as required for each reaction), 0.3 μL Taq polymerase (Invitrogen Canada Inc., Burlington, ON) or Vent polymerase (New England Biolabs Ltd., Pickering, ON), and volume up to 50 μL with sterile distilled water. Amplifications were performed with a hot start at 96°C for 6 min, 30 cycles of 94°C for 40 sec, 51.6°C-55°C for 40 sec, and 72°C for 1-2 min depending on primers, templates and sizes of expected products, followed by an extension step at 72°C for 10 min. The amplified PCR products were subsequently electrophoresed on a 0.8%-1% (w/v) agarose gel at 80-100 voltage according to the size of the PCR fragments.

Table 2-1. Vectors used in this study

Vector name (Source)	**Relevant Characteristics**	**Selectable Makers in *E. coli* host (μg/mL)**
pBluescript SKII(-) (Invitrogen)	Cloning vector with multiple cloning sites	Amp100
pGEM-T (Promega)	Cloning vector with multiple cloning sites	Amp100
pNAP-rbc-GEM-T (Alberta Research Council, Vegreville)	Modified pGEM-T vector with seed-specific Napin promoter and rbcS3' terminator insertions upstream and downstream of multiple cloning sites region, flanked by NarI restriction sites (Figure 2-7c)	Amp100
pKYLX-NAP (Alberta Research Council, Vegreville)	Plant binary gene expression vector with seed-specific Napin promoter and *nptII* gene for Kanamycin selectable marker in plant (Figure 2-7a)	Tet15
pRD320 (Plant Biotechnology Institute, Saskatoon)	Plant binary gene expression vector with *nptII* and *pat* genes for Kanamycin and PPT herbicide selectable markers in plant (Figure 2-15a)	Kan50

2.2.6 DNA digestion and ligation

DNA digestions were performed using several restriction endonucleases, supplied by NEB and Invitrogen. The reactions were carried out at 37ºC for a sufficient length of time as required for each reaction. Digested products were electrophoresed on 0.8%-1.0% (w/v) agarose gel to confirm a complete digestion and to identify sizes of the digested products. Ligation reactions were performed using T4 ligase supplied by Invitrogen. The reactions were carried out at 14ºC overnight, followed by transformation into competent *E. coli* DH5α cells (refer to 2.2.9).

2.2.7 DNA purification

PCR products and products from restriction reactions were purified using two methods. The products from enzymatic reactions were cleaned up using the PCR purification Kit (Qiagen). Reaction solution was passed through a spin column by centrifugation along with the binding buffer with high salt concentration. DNA in the sample was absorbed to the silica gel in the column, while impurities went through. After washed twice with wash buffer provided, pure DNA sample was eluted in a small amount of sterile distilled water.

DNA fragments after gel-electrophoresis were purified using the Gel-purification Kit (Qiagen). Gel slice containing target DNA fragment was dissolved in a buffer with high salt concentration at 55ºC for 10 min, and loaded into a spin column. The subsequent procedures were the same as described for PCR purification Kit.

2.2.8 DNA partial digestion

Plasmid DNA was digested in serial dilutions of restriction endonuclease in five separate tubes (2/3, 2/9, 2/27, 2/54, and 1/162 units of enzyme). The process of preparation was performed on ice in order to accurately control the reaction time. The mixture was incubated at 37°C for 30 min, and immediately returned on ice. The reaction solution was gel-electrophoresed on 1% (w/v) agarose gel to identify sizes of digested products.

2.2.9 Transformation of competent *E. coli* DH5α cells

2 μL of a ligation reaction was mixed with a 50 μL aliquot of competent *E. coli* DH5α cells (Invitrogen), which was previously thawed on ice. The mixture was incubated on ice for 20 min, followed by a heat shock at 42°C for 90 sec, and a cold shock on ice for 2 min. Pre-warmed 1 mL of LB at 37°C was added to the cell mixture, and incubated at 37°C for 1 hour 30 min. The suspension was plated on LB plates with appropriate antibiotics. The plates were incubated overnight at 37°C.

2.2.10 Plasmid DNA extraction

E. coli cells were harvested from 5-10 mL of liquid culture by centrifugation at 4,000 x g, for 10 min at room temperature (RT) after shaking at 37°C overnight with appropriate antibiotics. Plasmid DNA was extracted using Miniprep Extraction Kit (Qiagen). The bacterial culture was lysed with high-salted buffer, and centrifuged to remove cell debris. The cleared lysate was then applied to a spin column. After centrifugation, the plasmid DNA was captured onto the silica gel membrane in the column, while other impurities were washed away by wash buffer. The pure plasmid DNA was eluted in a small volume of elution buffer (20-50 μL) with low salt concentration or in sterile distilled water.

2.2.11 DNA sequencing

Premixed DNA samples with appropriate primers (0.1 μg per 1 kb of plasmid DNA and 3.2 pmol of primer in a final volume of 12 μL) were sent to University Core DNA Services (University of Calgary) for sequencing to be performed.

2.3 Results and Discussion

2.3.1 Isolation of *phb* genes and chloroplast transit peptide sequence (CTP)

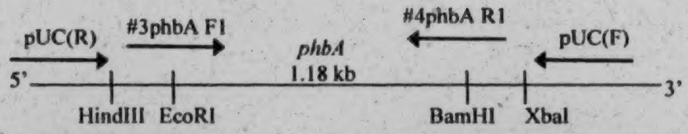
Three essential genes responsible for PHB biosynthesis (*phbA*, *phbB* and *phbC*) were isolated from *phbCAB* operon of *R. eutropha* H16 by colony PCR. A chloroplast transit peptide sequence, which encodes a target signal peptide, was isolated as well by

PCR using genomic DNA of *A. thaliana* as a template. All of the amplified DNA fragments were sub-cloned into the cloning vector pBluescript SKII(-) for sequencing and further manipulations.

2.3.1.1 Isolation of *phbA* gene from *R. eutropha* H16

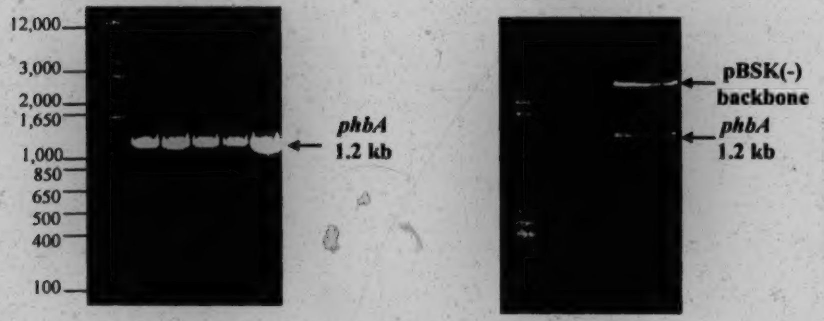
phbA gene, which encodes β-ketothiolase, was isolated from *R. eutropha* H16 by colony PCR with Vent polymerase, using a forward primer named "#3phbA F1" (5'-GCGC**GAATTC**ACA*ATG*ACTGACGTTGTC-3') with an EcoRI restriction site and a reverse primer named "#4phbA R1" (5'-AGTA**GGATCC***TTA*TTTGCGCTCGACT GCC-3') with a BamHI restriction site (Figure 2-3a). The EcoRI and BamHI restriction sites, shown in bold letters, were engineered at the 5' end immediately upstream of the start codon, and at the 3' end immediately downstream of the stop codon, respectively. The start and stop codons are shown in underlined italics. The PCR reaction was carried out with the mixture of $[Mg^{2+}]$ 3 mM, glycerol 10% (v/v), and 2 night-grown *R. eutropha* H16 colonies at [96°C 6 min (94°C 40 sec, 51.6°C 40 sec, 72°C 1 min) x30, 72°C 10 min, 4°C ∞] cycle. Amplified PCR product showed bands of a size between 1.0-1.6 kb on an electrophoresis gel according to the marker bands. The size of the product is correspondent to the size of known *phbA* sequence (Peoples and Sinskey 1989b). (Figure 2-3b)

The *phbA* gene belongs to *phbCAB* operon (Gene bank#J04987, Peoples et al. 1989b); with 1,180 bp that encodes 394 amino acids. The amplified product was gel-purified and double-digested with EcoRI and BamHI endonucleases, to ligate into pBluescript SKII(-) cloning vector by the same restriction sites. Transformation of the plasmid DNA into *E. coli* DH5α was confirmed by both blue-white selections with X-gal on LB agar plates containing Ampicillin (100 μg/mL), and by colony PCR with Taq polymerase using the same primers described. A few white colonies with the proper size of colony PCR product were inoculated in LB liquid media with Ampicillin selection for plasmid DNA extraction. Plasmid DNA was extracted on the following day for restriction analysis with EcoRI and BamHI digestion (Figure 2-3c). The clone, which showed the presence of an insert of proper size, was then sequenced with "pUC (Reverse)" and "pUC



a. Diagram of primers and restriction sites

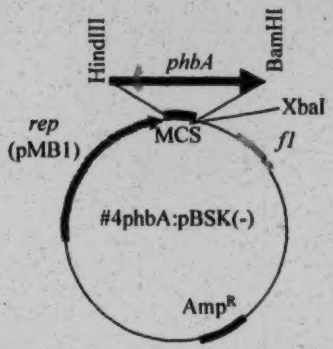
pUC(R)
#3phbA F1
phbA
1.18 kb
#4phbA R1
pUC(F)
5'
3'
HindIII
EcoRI
BamHI
XbaI



b. *phbA* isolation by colony PCR **c. Restriction analysis of #4phbA:pBSK(-)**

12,000
3,000
2,000
1,650
1,000
850
650
500
400
100
phbA
1.2 kb
pBSK(-)
backbone
phbA
1.2 kb

d. Plasmid map for #4phbA:pBSK(-)



HindIII
phbA
BamHI
XbaI
rep
(pMB1)
MCS
f1
#4phbA:pBSK(-)
AmpR

Figure 2-3. Isolation of *phbA* from *R. eutropha*

a.) arrows indicate primers used in *phbA* isolation and sequencing. EcoRI and BamHI restriction sites were incorporated into "#3phbA F1" forward and "#4phbA R1" reverse primers at the 5' and 3' ends of the sequences. b.) colony PCR product showed isolated *phbA* fragment from *R. eutropha*. The arrow indicates *phbA* bands of 1.2 kb size on an electrophoresis gel. Sizes of DNA molecular marker (1 kb Plus DNA ladder, Invitrogen, which has been used throughout this thesis) are shown in bp c.) restriction analysis with EcoRI and BamHI digestion on #4phbA:pBSK(-) plasmid. Arrows indicate *phbA* insert of 1.2 kb and pBluescript SKII(-) backbone. d.) #4phbA:pBSK(-) plasmid map, which contains *phbA* insert in multiple cloning sites (MCS) of pBluescript SKII(-) vector, and an Ampicillin resistance fragment (Amp^R).

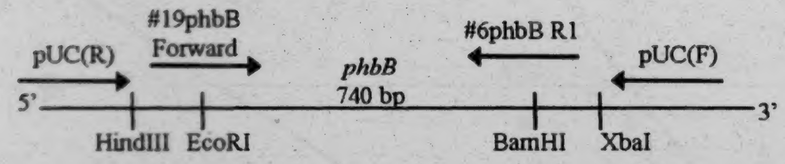
(Forward)" sequencing primers (Figure 2-3a). Comparison of the sequence to the known *phbA* sequence confirmed that the isolated gene had no mismatch. This pBluescript SKII(-) carrying *phbA* insert with the correct sequence and the engineered restriction sites was named #4phbA:pBSK(-) (Figure 2-3d). The bacterial clone was then re-cultured and stored at -80°C.

2.3.1.2 Isolation of *phbB* gene from *R. eutropha* H16

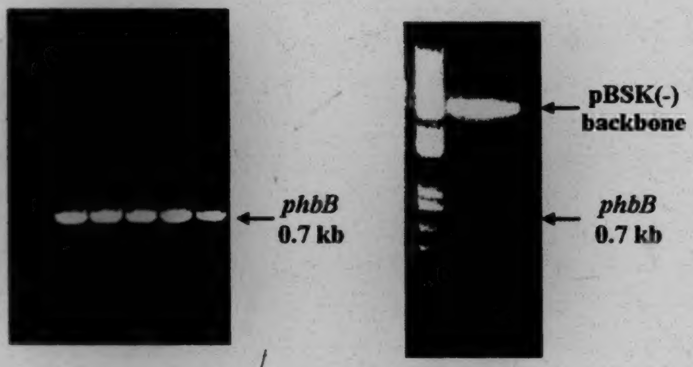
phbB gene, which encodes acetoacetyl-CoA reductase, was isolated from *R. eutropha* H16 by colony PCR with Vent polymerase, using a forward primer named "#19phbB Forward" (5'-AGTCA**GAATTC***ATG*ACTCAGCGCATTG-3') with an EcoRI restriction site and a reverse primer named "#6phbB R1" (5'-GGATTA**GGATCC**GCAGG*TCA*GCCCATATGC-3') with a BamHI restriction site (Figure 2-4a). The EcoRI and BamHI restriction sites, shown in bold letters, were engineered at the 5' end immediately upstream of the start codon, and at the 3' end downstream of the stop codon, respectively. The start and stop codons are shown in underlined italics. The PCR reaction was carried out with the mixture of [Mg^{2+}] 3 mM, and 2 night-grown *R. eutropha* H16 colonies at [96°C 6 min (94°C 40 sec, 51.6°C 40 sec, 72°C 1 min) x30, 72°C 10 min, 4°C ∞] cycle. Amplified PCR product showed bands of a size between 650-850 bp, on an electrophoresis gel according to the marker bands. The size of the product corresponds to the size of the known *phbB* sequence (Peoples and Sinskey 1989b) (Figure 2-4b). Subsequent procedures were same as described in isolation of *phbA* gene.

The *phbB* gene belongs to *phbCAB* operon (Gene bank#J04987, Peoples and Sinskey 1989b), with 738 bp that encodes 247 amino acids. The amplified product was gel-purified and double-digested with EcoRI and BamHI endonucleases, to ligate into pBluescript SKII(-) cloning vector by the same restriction sites. Transformation of the plasmid DNA into *E. coli* DH5α was confirmed by both blue-white selections with X-gal on LB agar plates containing Ampicillin (100μg/mL), and by colony PCR with Taq polymerase using the same primers. A few white colonies with the proper size of colony PCR products were inoculated in LB liquid media with Ampicillin selection for plasmid DNA extraction. Plasmid DNA was extracted on the following day for restriction

a. Diagram of primers and restriction sites


#19phbB
Forward
pUC(R)
#6phbB R1
pUC(F)
phbB
740 bp
5'
3'
HindIII
EcoRI
BamHI
XbaI

b. *phbB* isolation by colony PCR **c. Restriction analysis of #1phbB:pBSK(-)**


phbB
0.7 kb
pBSK(-)
backbone
phbB
0.7 kb

d. Plasmid map for #1phbB:pBSK(-)

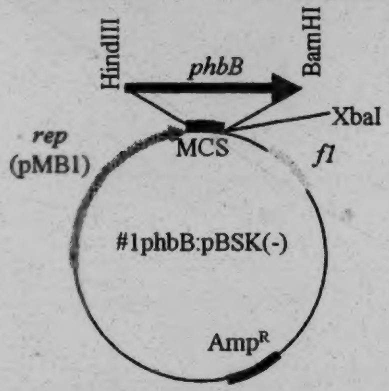

HindIII
phbB
BamHI
XbaI
rep
(pMB1)
MCS
f1
#1phbB:pBSK(-)
AmpR

Figure 2-4. Isolation of *phbB* from *R. eutropha*
a.) arrows indicate primers used in *phbB* isolation and sequencing. EcoRI and BamHI restriction sites were incorporated into #19phbB Forward" forward and "#6phbB R1" reverse primers at the 5' and 3' ends of the sequences. b.) colony PCR product showed isolated *phbB* fragment from *R. eutropha*. The arrow indicates *phbB* bands of 0.7 kb size. c.) restriction analysis with EcoRI and BamHI digestion on #1phbB:pBSK(-) plasmid. Arrows indicate *phbB* insert of 0.7 kb and pBluescript SKII(-) backbone. d.) #1phbB:pBSK(-) plasmid map, which contains *phbB* insert in multiple cloning sites (MCS) of pBluescript SKII(-) vector, and an Ampicillin resistance gene cassette (Amp^R).

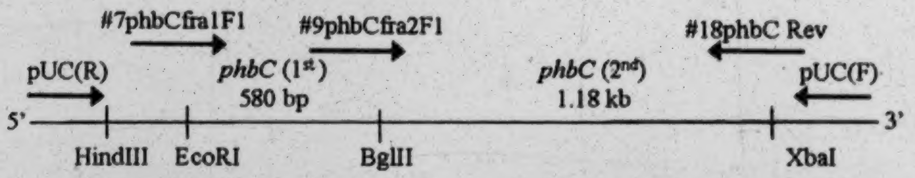
analysis with EcoRI and BamHI digestion (Figure 2-4c). The clone, which had an insert of correct size, was sequenced with “pUC (Reverse)” and “pUC(Forward)” sequencing primers to confirm no mismatch (Figure 2-4a). This pBluescript SKII(-) carrying *phbB* insert of correct sequence was named #1phbB:pBSK(-) (Figure 2-4d). The bacterial clone was then re-cultured and stored at -80°C.

2.3.1.3 Isolation of *phbC* gene from *R. eutropha* H16

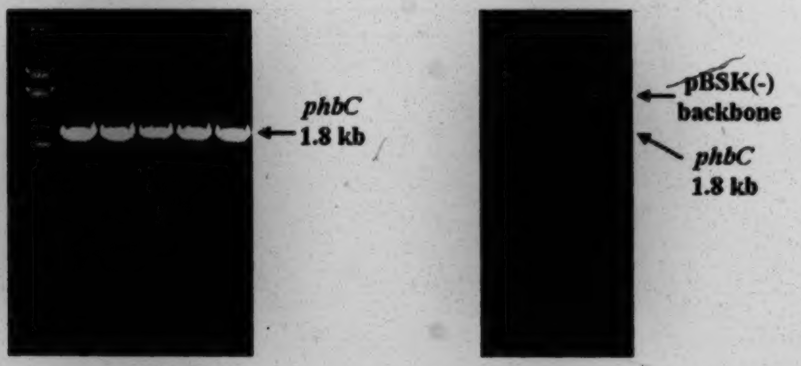
phbC gene, which encodes PHA synthase, was isolated from *R. eutropha* H16 by colony PCR with Vent polymerase as described above, using a forward primer named “#7phbCfra1F1” (5’-AACAT**GAATTC***ATG*GCGACCGGCAAAGG-3’) with an EcoRI restriction site and a reverse primer named “#18phbC Reverse” (5’-CTA**TCTAGA**AAGCG*TCA*TGCCTTGGC-3’) with an XbaI restriction site (Figure 2-5a). The EcoRI and XbaI restriction sites, shown in bold letters, were engineered at the 5’ end immediately upstream of the start codon, and at the 3’ end downstream of the stop codon respectively. The start and stop codons are shown in underlined italics. PCR mixture and reaction condition were the same as used to clone *phbA* and *phbB*, except the extension time was 1 min 50 sec. Amplified PCR product showed bands of a size between 1.6-2.0 kb on an electrophoresis gel, corresponding to the size of the known *phbC* sequence (Peoples and Sinskey 1989a) (Figure 2-5b).

The *phbC* gene belongs to *phbCAB* operon (Gene bank#J05003, Peoples and Sinskey 1989a), with 1,767 bp, which encodes 589 amino acids. The amplified product was gel purified, double-digested with EcoRI and XbaI endonucleases, ligated into pBluescript SKII(-), and transformed into *E. coli* DH5α, as described in isolations of *phbA* and *phbB* genes. A few recombinant clones carrying an insert with correct size were then sequenced with “pUC (Reverse)”, “#7phbCfra1R1”, “#9 phbCfra2F1”, “pUC(Forward)” primers (Figure 2-5a). No clone with a completely correct sequence was found. Hence two correct fragments were taken out of two separate clones and ligated together with an existing BglII restriction site present in the middle of *phbC* sequence (Figure 2-5a). The first fragment of 580 bp was taken from clone #4, while the second fragment of 1,187 bp was taken from clone #3.

a. Diagram of primers and restriction sites


#7phbCfra1F1
#9phbCfra2F1
#18phbC Rev
pUC(R)
phbC (1st)
580 bp
phbC (2nd)
1.18 kb
pUC(F)
5'
3'
HindIII
EcoRI
BglII
XbaI

b. *phbC* isolation by colony PCR **c. Restriction analysis of #2phbC:pBSK(-)**


phbC
1.8 kb
pBSK(-)
backbone
phbC
1.8 kb

d. Plasmid map for #2phbC:pBSK(-)

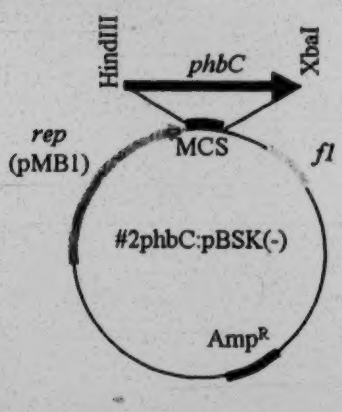

HindIII
phbC
XbaI
rep
(pMB1)
MCS
f1
#2phbC:pBSK(-)
AmpR

Figure 2-5. Isolation of *phbC* from *R. eutropha*
a.) arrows indicate primers used in *phbC* isolation and sequencing. EcoRI and XbaI restriction sites were incorporated into "#7phbCfra1F1" forward and "#18phbC Rev" reverse primers at the 5' and 3' ends of the sequences. b.) colony PCR product showed isolated *phbC* from *R. eutropha*. The arrow indicates *phbC* bands of 1.8 kb size. c.) restriction analysis with EcoRI and XbaI digestion on #2phbC:pBSK(-). Arrows indicate *phbC* insert of 1.8 kb and digested pBluescript SKII(-) backbone. d.) #2phbC:pBSK(-) plasmid map, which contains *phbC* insert in multiple cloning sites (MCS) of pBluescript SKII(-) vector, and an Ampicillin resistance gene cassette (Amp^R).

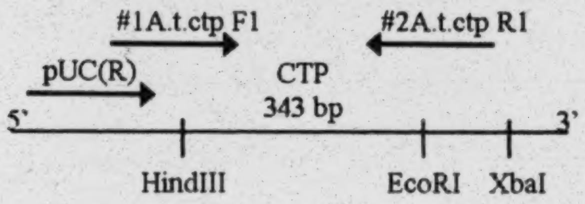
Both fragments were ligated and inserted into one pBluescript SKII(-). Transformation of the plasmid DNA into *E. coli* DH5α cells was confirmed by colony PCR with Taq polymerase. Restriction analysis was performed by double-digestion with HindIII and XbaI (Figure 2-5c). Clone #2, which had the proper size insert, was named #2phbc:pBSK(-) (Figure 2-5d). The plasmid DNA was sequenced with the same sequencing primers to confirm the full-length correct sequence. Comparison of the sequence to the known *phbC* sequence confirmed no mismatch. The bacterial clone was then re-cultured and stored at -80°C.

2.3.1.4 Isolation of chloroplast transit peptide sequence (CTP) from *A. thaliana*

Chloroplast transit peptide sequence (CTP) was isolated from genomic DNA extracted from leaf tissue of *A. thaliana*, variety WS, by PCR amplification with Taq polymerase, using a forward primer named "#1A.t.ctp F1" (5'–GCGC**AAGCTT***ATG*GCTTCCTCTATGCTCT–3') with a HindIII restriction site and a reverse primer named "#2A.t.ctp R1" (5'–GTCC**GAATTC**CAATTCGGAATCGGTAAGG–3') with an EcoRI (Figure 2-6a). The HindIII and EcoRI restriction sites are shown in bold letters. The HindIII site was engineered at the 5' end, immediately upstream of the start codon, while the EcoRI site is naturally present in the CTP fragment after codons of 24 mature amino acids (refer to 2.1.3). The PCR reaction was carried out with the mixture of genomic DNA template 15 μL, [Mg^{2+}] 3 mM, at [96°C 6 min (94°C 40 sec, 51.6°C 40 sec, 72°C 1 min) x30, 72°C 10 min, 4°C ∞] cycle. Amplified PCR produced a band of correct size between 300-400 bp on an electrophoresis gel. The size of the product corresponds to the size of the known CTP sequence (Wong et al. 1992) (Figure 2-6b).

The CTP fragment contains 343 bp (237 bp exon and 106 bp intron), which encodes 55 amino acids of transit peptide and first 24 amino acids of Rubisco small subunit mature protein (Gene Bank#NM_202369, Wong et al. 1992). The amplified product was gel-purified and double-digested with HindIII and EcoRI, to ligate into pBluescript SKII(-) cloning vector, and transformed into *E. coli* DH5α. Plasmids that showed correct size insert by colony PCR and restriction analysis were sequenced with "pUC (Reverse)" sequencing primer (Figure 2-6a). Comparison with the known CTP

a. Diagram of primers and restriction sites


#1A.t.ctp F1
#2A.t.ctp R1
pUC(R)
CTP
343 bp
5'
3'
HindIII
EcoRI
XbaI

b. CTP isolation by genomic DNA PCR **c. Plasmid map for #1CTP:pBSK(-)**

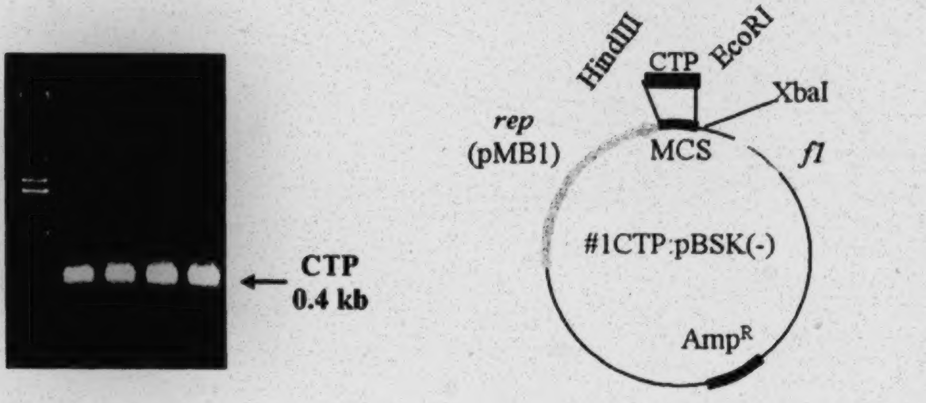
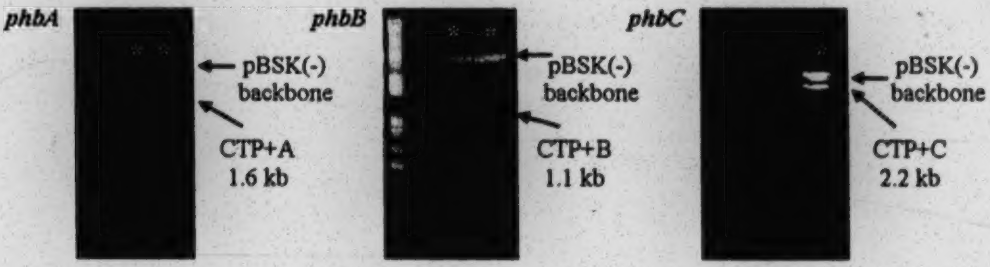
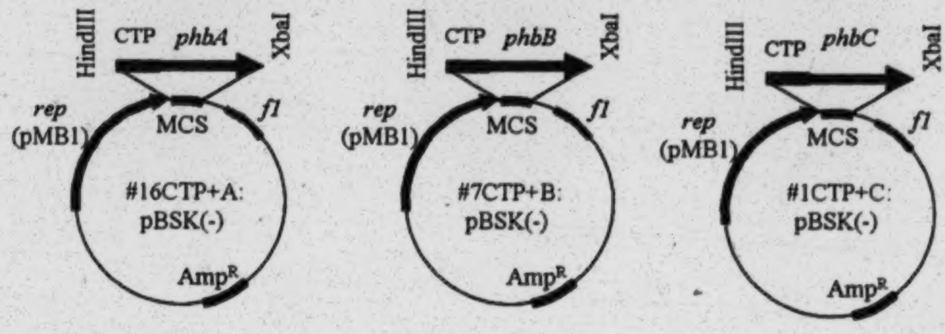

CTP
0.4 kb
HindIII
CTP
EcoRI
XbaI
rep
(pMB1)
MCS
f1
#1CTP:pBSK(-)
AmpR

Figure 2-6. Isolation of chloroplast transit peptide sequence (CTP) from *A. thaliana* and attachment to *phb* genes a.) arrows indicate primers and restriction sites used in CTP isolation and sequencing. HindIII and EcoRI restriction sites were incorporated into "#1A.t.ctp F1" forward and "#2A.t.ctp R1" reverse primers at the 5' and 3' ends of the CTP sequence. b.) amplified PCR product using *A. thaliana* genomic DNA template showed isolated CTP fragment. The arrow indicates CTP bands of 0.4 kb size. c.) #1CTP:pBSK(-) plasmid carrying CTP fragment in multiple cloning sites (MCS) of pBluescript SKII(-) vector, and an Ampicillin resistance gene cassette (Amp^R).

d. Restriction analysis of #16CTP+A:pBSK(-), #7CTP+B:pBSK(-), and #1CTP+C:pBSK(-)


phbA
pBSK(-) backbone
CTP+A 1.6 kb
phbB
pBSK(-) backbone
CTP+B 1.1 kb
phbC
pBSK(-) backbone
CTP+C 2.2 kb

e. Plasmid maps for #16CTP+A:pBSK(-), #7CTP+B:pBSK(-), and #1CTP+C:pBSK(-)


HindIII
CTP
phbA
XbaI
rep
(pMB1)
MCS
f1
#16CTP+A:
pBSK(-)
Amp^R
HindIII
CTP
phbB
XbaI
rep
(pMB1)
MCS
f1
#7CTP+B:
pBSK(-)
Amp^R
HindIII
CTP
phbC
XbaI
rep
(pMB1)
MCS
f1
#1CTP+C:
pBSK(-)
Amp^R

d.) restriction analysis with HindIII and XbaI digestion on #16CTP+A:pBSK(-), #7CTP+B:pBSK(-), and #1CTP+C:pBSK(-) plasmids. In electrophoresis gel pictures, arrows indicate CTP with *phbA* 1.6 kb (CTP 0.4 kb, *phbA* 1.2 kb), CTP with *phbB* 1.1 kb (CTP 0.4 kb, *phbB* 0.7 kb), CTP with *phbC* 2.2 kb (CTP 0.4 kb, *phbC* 1.8 kb), and digested pBluescript SKII(-) backbones. e.) #16CTP+A:pBSK(-), #7CTP+B:pBSK(-), and #1CTP+C:pBSK(-) plasmids contain CTP fragment attached to *phbA*, *phbB* and *phbC* respectively. The *phb* genes with CTP were inserted in multiple cloning sites (MCS) of pBluescript SKII(-) vector carrying an Ampicillin resistance gene cassette (Amp^R).

sequence found one mutation at position 196 from C to T. This mutation occurred in the first intron region further apart from the first exon splice site by 25nts and from the second exon by 80nts. Since the mutation was in the middle of the intron region, which would be spliced out before RNA processing, it should not have any influence on the polypeptide sequence produced by the mRNA template. This pBluescript SKII(-) carrying a CTP insert with the engineered restriction sites was named #1CTP:pBSK(-) (Figure 2-6c). The bacterial clone was then re-cultured and stored at -80°C.

2.3.2 Gene Constructs for Seed-specific Expressions

Isolated *phb* genes and chloroplast transit peptide fragment in separate pBluescript SKII(-) cloning vectors were subsequently sub-cloned into plant binary expression vectors. Two sets of gene constructs were prepared, driven by seed-specific promoters (Napin promoter) to introduce PHB synthetic pathway into developing seeds. The first gene construct was a double-cassette construct carrying *phbB* and *phbC* expressed in cytosolic compartments in the developing seeds. The second set is comprised of 2 gene constructs, a double-cassette construct containing *phbA* and *phbB*, and a single gene construct carrying *phbC*. Each of the three transgenes has a CTP fragment attached to the 5' end upstream of the start codon in the same reading frame, to target the gene expression to leucoplast sub-cellular compartments. As explained in 2.1.2, expression of β-ketothiolase is naturally found in cytosol of higher plants, therefore the production of PHB in cytoplasm only requires expressions of *phbB* and *phbC* genes. PHB production in leucoplast, on the other hand requires all three gene expressions with the attachment of chloroplast transit peptide to each of them.

2.3.2.1 *phbB* and *phbC* double-cassette construct for cytosolic expression

Full-length *phbC* fragment was taken out of #2phbc:pBSK(-) plasmid (refer to 2.3.1.3) by HindIII and XbaI double-digestion (Figure 2-5a). The fragment was ligated into pKYLX-NAP expression vector by the same restriction sites in the multiple cloning region. Transformed *E. coli* DH5α cells were plated on LB agar plates with Tetracycline (15 μg/mL) selection. Two colonies were randomly picked up from the plate and cultured in LB liquid media with Tetracycline selection for plasmid DNA extraction. Plasmid DNA was extracted from each colony, and restriction analysis was performed by double digestion with HindIII and XbaI. Both clones showed a proper sized-insert band of 1.8 kb. Clone #1 was selected and named #1phbC:pKYLX-NAP (Figure 2-7b), re-cultured and stored at -80C.

phbB fragment was taken out of the #1phbB:pBSK(-) plasmid (refer to 2.3.1.2) by HindIII and XbaI double digestions (Figure 2-4a). The fragment was ligated into pNAP-rbc-GEM-T modified cloning vector by the same restriction sites in the multiple cloning region. Transformed *E. coli* DH5α cells were plated on LB agar plate with Ampicillin (100 μg/mL) selection. A few colonies were picked and the presence of *phbB* insert was confirmed by colony PCR with Taq polymerase using "#19phbB Forward" and "#6phbB R1" primers (Figure 2-4a). Some of the colonies showed amplified PCR products of proper size, corresponding to the size of *phbB* insert. Clone #1 was selected and named #1phbB: pNAP-rbc-GEM-T (Figure 2-7d), re-cultured and stored at -80°C.

pNAP-rbc-GEM-T is a modified cloning vector, which has both the seed-specific Napin promoter and the rbcS3' terminator sub-cloned from pKYLX-NAP vector with multiple cloning sites in between. The whole insert is also flanked by two engineered NarI restriction sites upstream and downstream of the promoter and the terminator, respectively (Figure 2-7c). By utilizing this modified cloning vector, double-cassette gene constructs were prepared as described below.

Since a NarI restriction site is present in the middle of the *phbB* fragment, #1phbB:pNAP-rbc-GEM-T plasmid was partially digested at 37°C for 30 minutes, in order to take out the whole *phbB* gene cassette (Figure 2-8). The reaction solution was electrophoresed on a 1% agarose gel, to separate bands of different sizes. Band with a size of 2.5 kb, which was expected to be the entire *phbB* gene cassette (Napin promoter 1.1 kb, *phbB* 0.7 kb, rbcS3' terminator 0.7 kb, Figure 2-7d) were excised and gel-purified as for a preparation of an insert fragment.

#1phbC:pKYLX-NAP plasmid was digested with ClaI endonulease for the backbone preparation. The plasmid was cleaved at a restriction site downstream of the

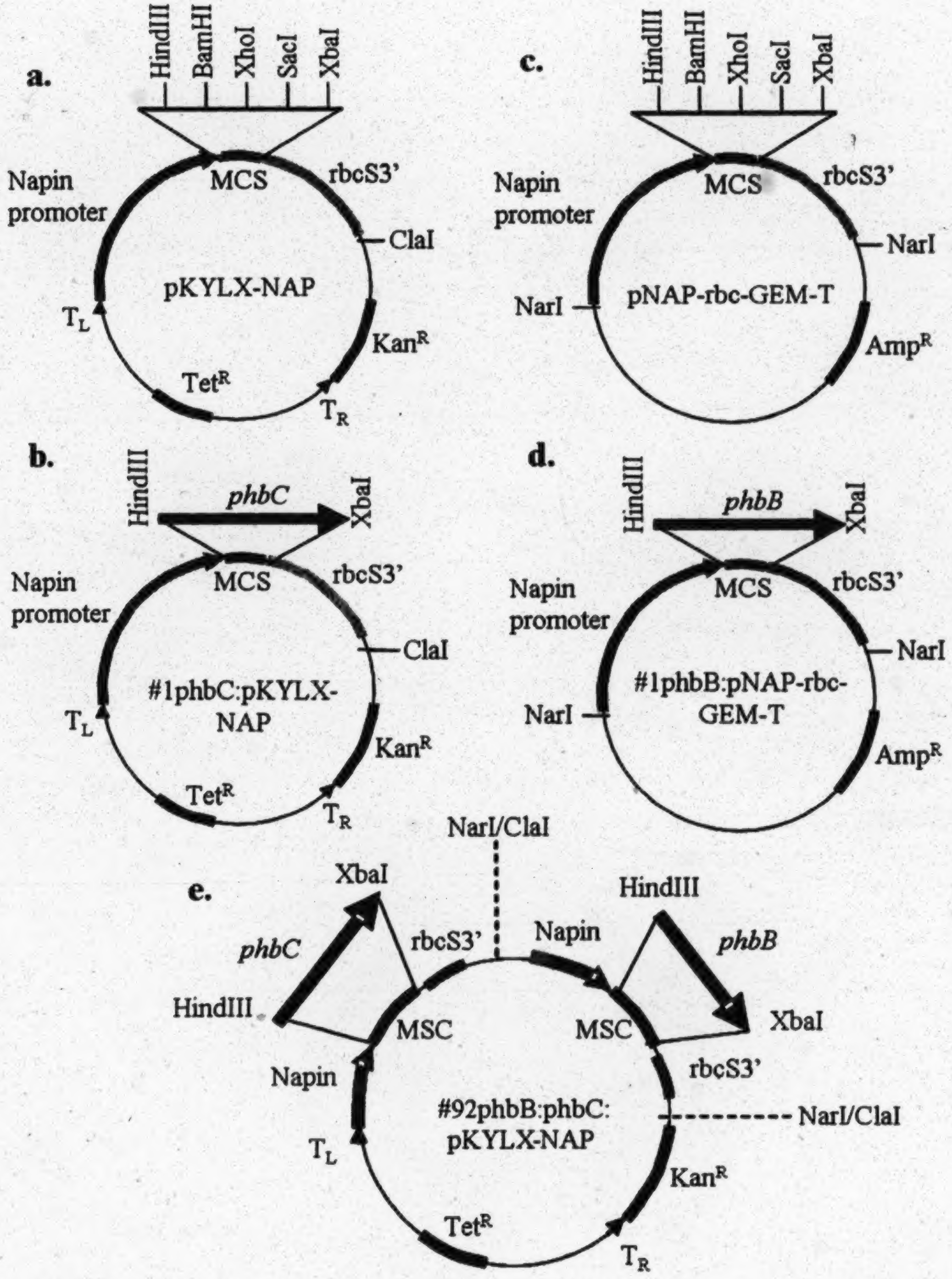

a.
HindIII
BamHI
XhoI
SacI
XbaI
Napin promoter
MCS
rbcS3'
ClaI
pKYLX-NAP
T_L
Kan^R
Tet^R
T_R
c.
HindIII
BamHI
XhoI
SacI
XbaI
Napin promoter
MCS
rbcS3'
NarI
NarI
pNAP-rbc-GEM-T
Amp^R
b.
HindIII
phbC
XbaI
Napin promoter
MCS
rbcS3'
ClaI
#1phbC:pKYLX-NAP
T_L
Kan^R
Tet^R
T_R
d.
HindIII
phbB
XbaI
Napin promoter
MCS
rbcS3'
NarI
NarI
#1phbB:pNAP-rbc-GEM-T
Amp^R
e.
NarI/ClaI
XbaI
phbC
rbcS3'
Napin
HindIII
phbB
HindIII
MSC
MSC
XbaI
Napin
rbcS3'
#92phbB:phbC:pKYLX-NAP
NarI/ClaI
T_L
Kan^R
Tet^R
T_R

Figure 2-7. Preparation of *phbB* and *phbC* double-cassette construct
a.) pKYLX-NAP binary gene expression vector. The vector contains a seed-specific Napin promoter, multiple cloning sites (MCS) for gene insertion, rbcS3' terminator, a Kanamycin resistance marker (Kan^R) and a ClaI restriction site between Ti-DNA left and right boarders (T_L and T_R). Tetracycline resistance marker is located outside the boarders. b.) #1phbC:pKYLX-NAP vector. *phbC* was inserted into pKYLX-NAP by HindIII and XbaI restriction sites in multiple cloning region. c.) pNAP-rbc-GEM-T, modified p-GEM-T cloning vector with Ampicillin resistance. The vector contains two NarI restriction sites, which are upstream and downstream of Napin promoter and rbcS3' terminator, respectively. d.) #1phbB:pNAP-rbc-GEM-T vector. *phbB* was inserted into pNAP-rbc-GEM-T by HindIII and XbaI restriction sites between Napin promoter and rbcS3' terminator. e.) #92phbB:phbC:pKYLX-NAP, a *phbB* and *phbC* double-cassette construct. *phbB* gene cassette taken from #1phbB:pNAP-rbc-GEM-T by NarI restriction sites was inserted into #1phbC:pKYLX-NAP at the ClaI site downstream of the rbcS3' terminator. NarI and ClaI are compatible enzymes.

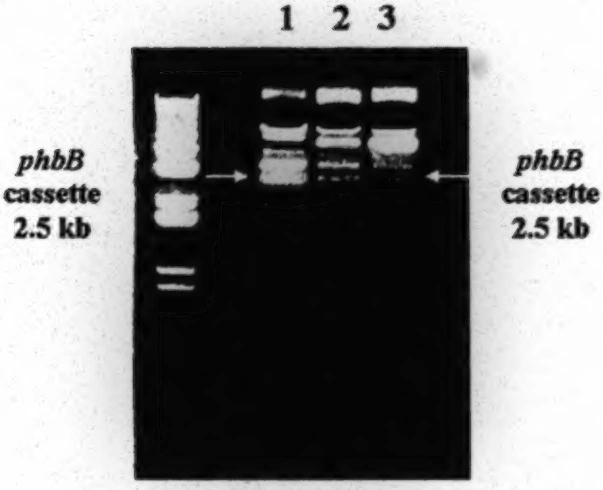


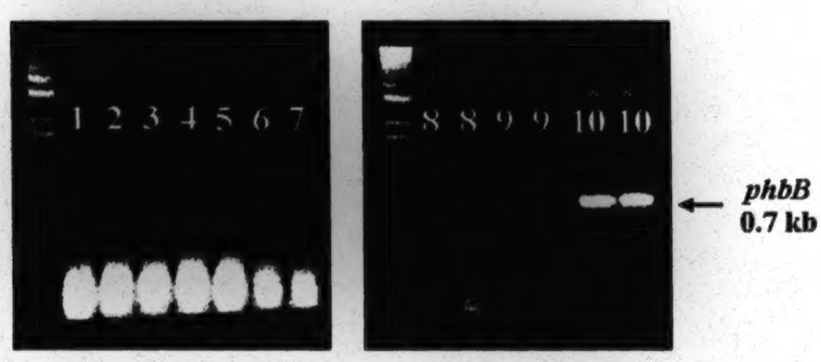
Figure 2-8. Partial digestion of *phbB* gene cassette from #1phbB:pNAP-rbc-GEM-T plasmid #1phbB:pNAP-rbc-GEM-T plasmid was digested with NarI at 37°C for 30 minutes and electrophoresed on a 1% agarose gel. Amounts of NarI endonuclease used in reactions 1, 2 and 3 were 2/3, 2/9 and 2/27 units, respectively. Arrows indicate *phbB* gene cassette bands with expected size of 2.5 kb to (Napin promoter 1.1 kb, *phbB* 0.7 kb, rbcS3' terminator 0.7 kb).

rbcS3' terminator (Figure 2-7b). The reaction solution was immediately used for ligation without any further purification. By taking advantage of the compatibility of NarI and ClaI restricted overhangs, the *phbB* cassette was inserted into digested #1phbC:pKYLX-NAP backbone by ligation overnight at 14°C. The plasmid DNA was transformed into *E. coli* DH5α host, and plated on LB agar plates with Tetracycline selection. Ninety-eight colonies were used as templates for pool-colony PCR; 10 colonies per PCR reaction with "#19phbB forward" and "#6phbB reverse" primers to identify the presence of the insert (Figure 2-9a). After confirmation of a positive band in pool #10, which had colonies #91 to #98 as a template, PCR using individual colonies was performed with the same primers. Clones #92 and #93 showed positive amplified product with the size corresponding to *phbB* (Figure 2-9b). To confirm the presence of full-length *phbC* in #92 and #93 clones, another colony PCR reaction was performed using "#7phbC forward" and "#10phbC reverse" primers. Both clones showed positive amplified PCR product with the size corresponding to *phbC* (Figure 2-9c). Additionally, restriction analysis was carried out by HindIII and XbaI double-digestion of plasmid DNA extracted from each clone, to confirm the presence of both insert genes (Figure 2-10a). The orientation of *phbB* cassette inserted was identified by digestion with XbaI. Digested product showed a band with a size of approximate 2.5 kb, which confirmed that *phbB* cassette, was inserted into #1phbC:pKYLX-NAP backbone in a tandem order (Figure 2-10b). If the *phbB* cassette was inserted in the reverse tandem orientation; the digested product with XbaI would be approximate 1.4 kb. (Figure 2-10c) The clone #92 was named #92phbB:phbC: pKYLX-NAP, re-cultured and stored at -80°C (Figure 2-7e).

2.3.2.2 Chloroplast transit peptide attachment to *phb* genes

In order to target the expression of a gene into leucoplast sub-cellular compartments in developing seeds, not only an expression of seed-specific promoter but also an expression of chloroplast transit peptide (CTP) is required. To achieve this goal, it is crucial to attach a chloroplast transit peptide sequence to the gene in the same reading frame upstream of the start codon. In preparation for further steps, each *phb* genes was individually sub-cloned into #1CTP:pBSK(-) modified cloning vector (refer to 2.3.1).

a. Pool-colony PCR for *phbB* amplification



b. Colony PCR of clones from pool 10 for *phbB* amplification

c. Colony PCR of clones 92 and 93 for *phbC* amplification

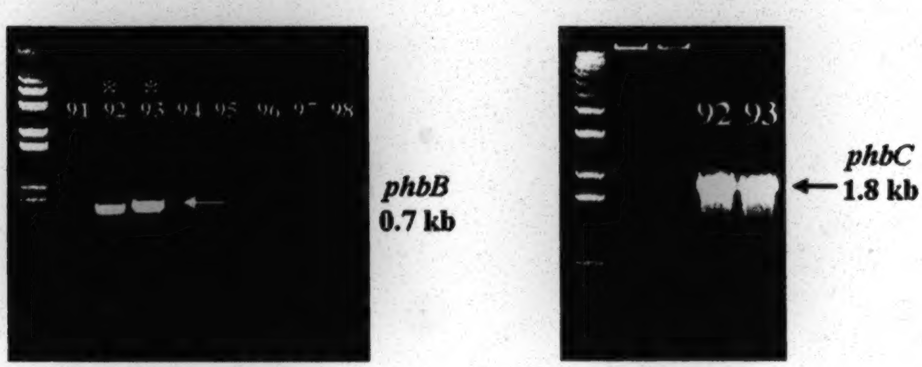
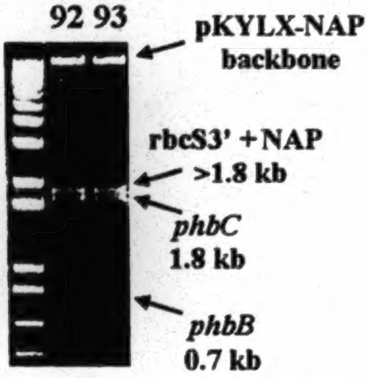
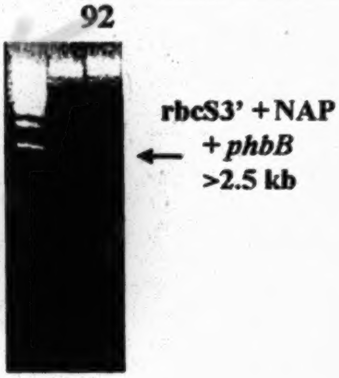


Figure 2-9. Identification of *phbB* and *phbC* genes by pool- and individual colony PCR a.) Pool-colony PCR of 98 colonies, obtained from transformation of ligation reaction of *phbB* gene cassette insert and #1phbC:pKYLX-NAP backbone. Ten colonies were used per PCR reaction with *phbB* primers. Number in each lane indicates the pool number. The arrow points at *phbB* positive bands of pool #10, which had colonies #91 to #98 as a template. b.) Colony PCR using individual colonies from pool #10 (#91 to #98) as a template. The arrow indicates amplified *phbB* bands of clones #92 and #93. c.) Colony PCR for *phbC* amplification using #92 and #93 clones as a template. The arrow points at positive *phbC* bands in both lanes.

a. Restriction analysis of #92 and #93phbB:phbC:pKYLX-NAP


92 93
pKYLX-NAP backbone
rbcS3' +NAP >1.8 kb
phbC 1.8 kb
phbB 0.7 kb

b. Restriction analysis of #92phbB:phbC:pKYLX-NAP for orientation of *phbB* cassette insertion


92
rbcS3' +NAP +*phbB* >2.5 kb

c. Diagrams of two orientations of *phbB* cassette insertion in #92phbB:phbC:pKYLX-NAP

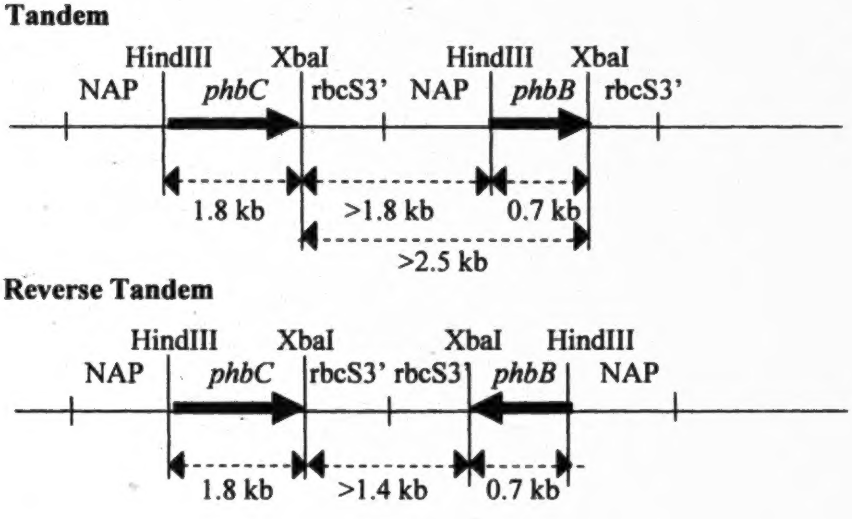

Tandem
HindIII
XbaI
HindIII
XbaI
NAP
phbC
rbcS3'
NAP
phbB
rbcS3'
1.8 kb
>1.8 kb
0.7 kb
>2.5 kb
Reverse Tandem
HindIII
XbaI
XbaI
HindIII
NAP
phbC
rbcS3'
rbcS3'
phbB
NAP
1.8 kb
>1.4 kb
0.7 kb

Figure 2-10. Restriction analysis of *phbB* and *phbC* genes, and orientation of *phbB* cassette insertion a.) restriction analysis of #92 and #92phbB:phbC:pKYLX-NAP plasmids with HindIII and XbaI digestion. Arrows indicate target *phbB* and *phbC* bands, as well as pKYLX-NAP backbone and rbcS3' with Napin promoter fragment with sizes corresponding to the diagram. b.) Restriction analysis of #92phbB:phbC:pKYLX-NAP with XbaI to identify orientation of *phbB* cassette. The arrow indicates digested fragment of 2.5 kb (rbcS3' 0.7 kb, Napin promoter 1.1 kb, *phbB* 0.7 kb), which confirmed a tandem order of *phbB* and *phbC* cassettes. c.) Diagrams show restriction sites and sizes of fragments of #92phbB:phbC:pKYLX-NAP in tandem and reverse tandem orders of *phbB* and *phbC* cassettes.

Full-length *phbA*, *phbB* and *phbC* gene fragments were taken out of #4phbA:pBSK(-), #1phbB:pBSK(-) and #2phbC:pBSK(-) plasmids respectively (refer to 2.3.1.1-3) by EcoRI and XbaI double-digestion. The fragments were individually inserted into the #1CTP:pBSK(-) backbone by the same restriction sites. Transformed *E. coli* DH5α cells were plated on LB agar plates with Ampicillin (100 μg/mL) selection. To confirm presence of each inserted gene, colony PCR reactions were performed using a few colonies from each transformation as a template with primers accordingly. Clones, which had shown the presence of the inserts, were randomly picked and cultured in LB liquid media with Ampicillin selection for plasmid DNA extraction. Plasmid DNA was extracted from each colony, and restriction analysis was performed by double digestion with HindIII and XbaI. All the clones showed insert of correct size: CTP with *phbA* 1.6 kb (CTP 0.4 kb, *phbA* 1.2 kb), CTP with *phbB* 1.1 kb (CTP 0.4 kb, *phbB* 0.7 kb), and CTP with *phbC* 2.2 kb (CTP 0.4 kb, *phbC* 1.8 kb) (Figure 2-6d). This result indicated that all of the genes were successfully fused with CTP in #1CTP:pBSK(-). Clone #16 of *phbA* insert was named as #16CTP+A:pBSK(-), clone #7 of *phbB* insert as #7CTP+B:pBSK(-), and clone #1 of *phbC* insert as #1CTP+C:pBSK(-). They were subsequently re-cultured and stored at -80°C (Figure 2-6e). After the presences of both CTP and insert genes were confirmed, it was important to ensure that the CTP fragment and the target genes were ligated to each other in the correct reading frame, so that the translation into amino acids would not be interrupted. Therefore the three plasmid vectors of *phb* genes with CTP attachement were sequenced. The reading frame of each vector was confirmed correct for translation into amino acids.

2.3.2.3 *phbA* and *phbB* double-cassette construct for plastidial co-transformation

A *phbA* and *phbB* double-cassette construct was prepared following the same strategy used to prepare the *phbB* and *phbC* double-cassette construct for cytosolic expression. *phbA* with CTP fragment was taken out of #16CTP+A:pBSK(-) plasmid by HindIII and XbaI double-digestion (Figure 2-6e). The fragment was then ligated into pKYLX-NAP expression vector by the same restriction sites in the multiple cloning region and transformed into *E. coli* DH5α. Colony #16, which carried recombinant

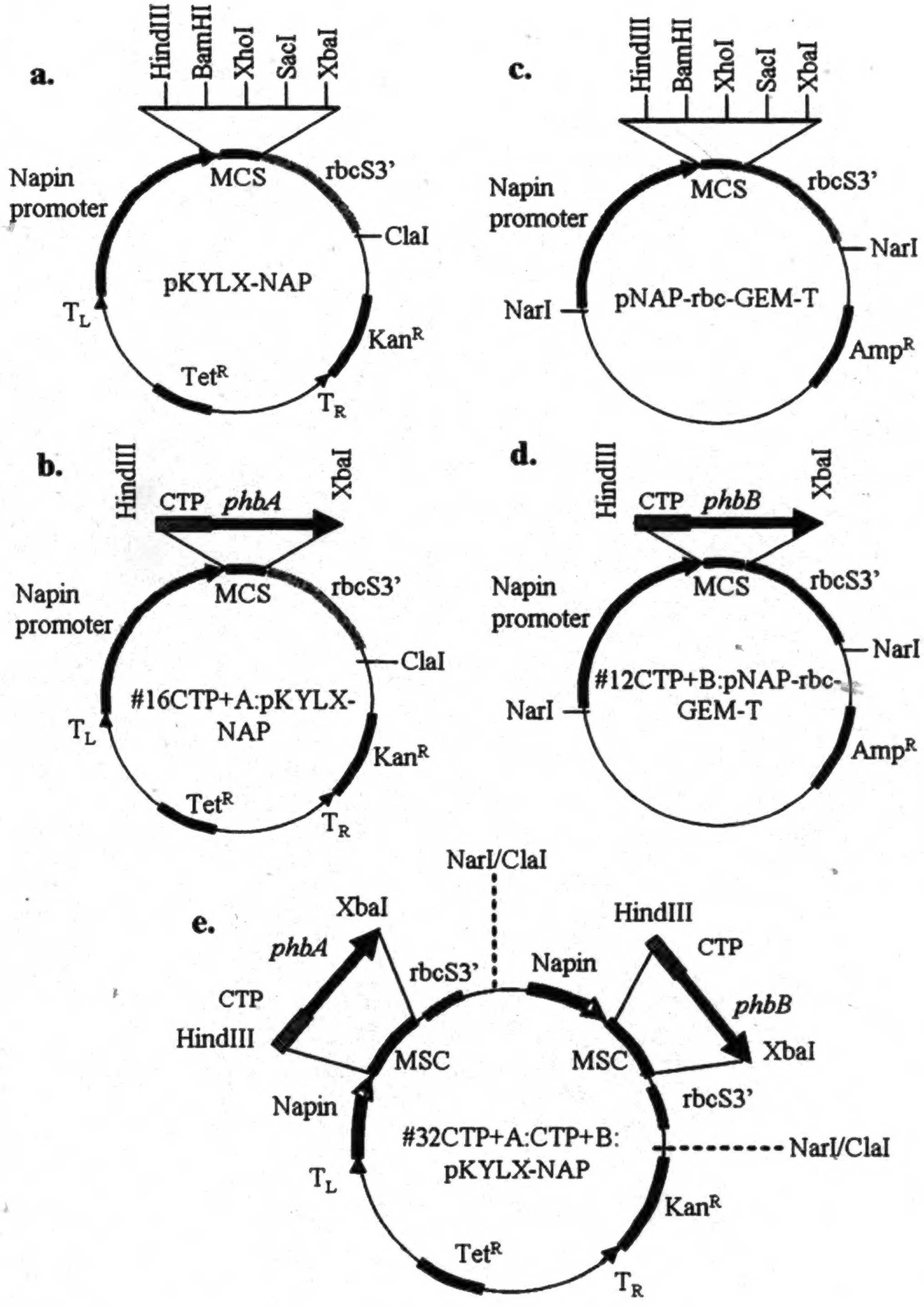

a.
HindIII
BamHI
XhoI
SacI
XbaI
Napin promoter
MCS
rbcS3'
ClaI
pKYLX-NAP
T_L
Kan^R
Tet^R
T_R
c.
HindIII
BamHI
XhoI
SacI
XbaI
Napin promoter
MCS
rbcS3'
NarI
NarI
pNAP-rbc-GEM-T
Amp^R
b.
HindIII
CTP
phbA
XbaI
Napin promoter
MCS
rbcS3'
ClaI
#16CTP+A:pKYLX-NAP
T_L
Kan^R
Tet^R
T_R
d.
HindIII
CTP
phbB
XbaI
Napin promoter
MCS
rbcS3'
NarI
NarI
#12CTP+B:pNAP-rbc-GEM-T
Amp^R
e.
NarI/ClaI
XbaI
phbA
CTP
HindIII
rbcS3'
Napin
HindIII
CTP
phbB
XbaI
MSC
MSC
Napin
rbcS3'
NarI/ClaI
#32CTP+A:CTP+B: pKYLX-NAP
T_L
Kan^R
Tet^R
T_R

Figure 2-11. Preparation of *phbA* and *phbB* double-cassette construct for plastidial co-transformation a.) pKYLX-NAP binary gene expression vector. The vector contains a seed-specific Napin promoter, multiple cloning sites (MCS) for gene insertion, rbcS3' terminator, a Kanamycin resistance marker (Kan^R) and a ClaI restriction site between left and right boarders (T_L and T_R). Tetracycline resistance marker is located outside the boarders. b.) #16CTP+A:pKYLX-NAP vector. *phbA* with CTP attachment was inserted into pKYLX-NAP by HindIII and XbaI restriction sites in multiple cloning region. c.) pNAP-rbc-GEM-T, modified p-GEM-T cloning vector with an Ampicillin resistance gene. The vector contains two NarI restriction sites, which are upstream and downstream of Napin promoter and rbcS3' terminator, respectively. d.) #12CTP+B:pNAP-rbc-GEM-T vector, *phbB* with CTP attachment was inserted into pNAP-rbc-GEM-T by HindIII and XbaI sites. e.) #32CTP+A:CTP+B:pKYLX-NAP, a *phbA* and *phbB* double-cassette construct. *phbB* with CTP gene cassette taken from #12CTP+B:pNAP-rbc-GEM-T by NarI restriction sites was inserted into #16CTP+A:pKYLX-NAP at the ClaI site downstream of the rbcS3' terminator. NarI and ClaI are compatible enzymes.

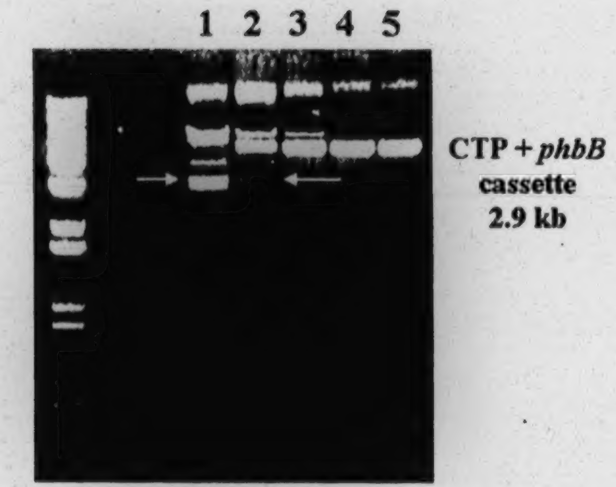
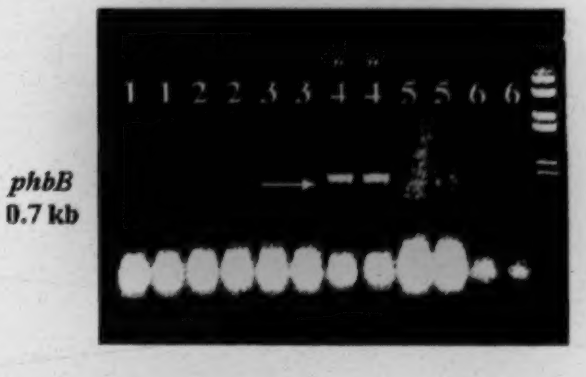


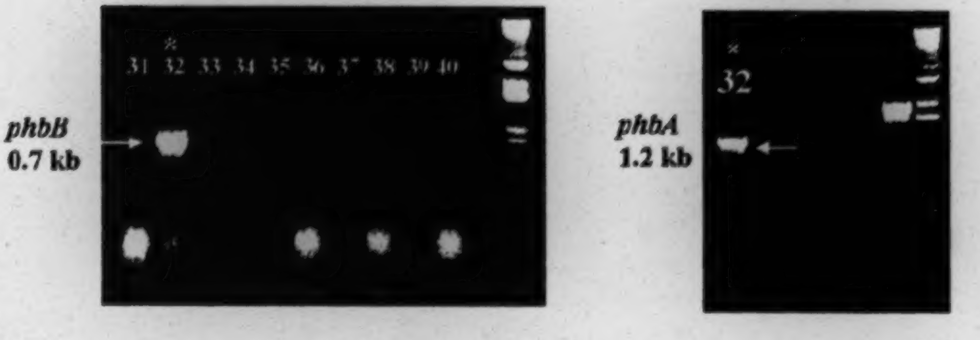
Figure 2-12. Partial digestion of *phbB* with CTP cassette from #12CTP+B:pNAP-rbc-GEM-T plasmid #12CTP+B:pNAP-rbc-GEM-T plasmid was digested with NarI endonuclease at 37°C for 30 minutes and electrophoresed on a 1% agarose gel. Amounts of NarI endonuclease used in reactions 1, 2, 3, 4 and 5 were 2/3, 2/9, 2/27, 2/54, and 1/162 units, respectively. Arrows indicate *phbB* with CTP cassette bands with expected size of 2.9 kb to (Napin promoter 1.1 kb, CTP 0.4 kb, *phbB* 0.7 kb, rbcS3' terminator 0.7 kb).

plasmid as tested by colony PCR, was used for plasmid DNA extraction. Restriction digestion by HindIII and XbaI showed gene product of correct size of 1.6kb (CTP 0.4 kb, *phbA* 1.2 kb). The clone was named #16CTP+A:pKYLX-NAP, re-cultured and stored at -80°C (Figure 2-11b).

Following the same process as preparation of #1phbB: pNAP-rbc-GEM-T, *phbB* with CTP fragment was taken out of #7CTP+B:pBSK(-) plasmid by HindIII and XbaI double-digestion (Figure 2-6e). The fragment was ligated into pNAP-rbc-GEM-T modified cloning vector by the same restriction sites. Transformation was confirmed by colony PCR to confirm fusion of *phbB* with CTP. Colony #12 with correct size amplified product of 1.1 kb (CTP 0.4 kb, *phbB* 0.7 kb) was named #12CTP+B: pNAP-rbc-GEM-T, re-cultured and stored at -80°C (Figure 2-11d). The complete *phbB* with CTP gene cassette was taken out off #12CTP+B: pNAP-rbc-GEM-T by partial digestion with NarI. The reaction solution was electrophoresed on a 1% agarose gel. The 2.9 kb bands of *phbB* with CTP cassette (Napin promoter 1.1 kb, CTP 0.4 kb, *phbB* 0.7 kb, rbcS3' terminator 0.7 kb) were excised and gel-purified as for a preparation of the insert fragment (Figure 2-12).

For backbone preparation, #16CTP+A:pKYLX-NAP plasmid was cleaved with ClaI at the site downstream of *nptII* cassette (Figure 2-11b). *phbB* with CTP cassette was ligated into cleaved #16CTP+A:pKYLX-NAP and transformed into *E. coli* DH5α. Fifty-five colonies were used as templates for pool-colony PCR to investigate the presence of *phbB* insert (Figure 2-13a). Pool #4 showed a positive band with correct size of 0.7 kb. PCR of colonies #31 to #40 were individually performed. Clones #32 showed a positive amplified product with size corresponding to *phbB* (Figure 2-13b). Plasmid DNA of clone #32 was used for colony PCR with *phbA* primers (Figure 2-13c), and for restriction analysis with HindIII and XbaI to confirm the presences of both genes (Figure 2-14a). The orientation of *phbB* cassette was identified by digestion with XbaI. Digested product showed a band with a size of approximate 2.9 kb, which confirmed that the cassette was introduced into #16CTP+A:pKYLX-NAP backbone in a tandem order (Figure 2-14b). If the cassette was inserted in the reverse tandem manner; the digested product would be of

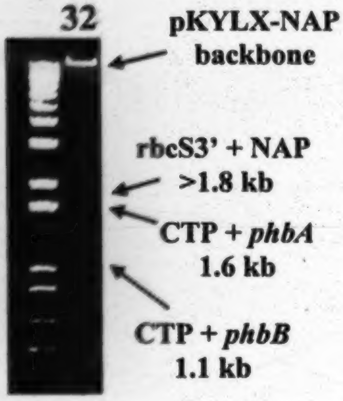

a. Pool-colony PCR for *phbB* amplification


b. Colony PCR of clones from pool 4 for *phbB* amplification

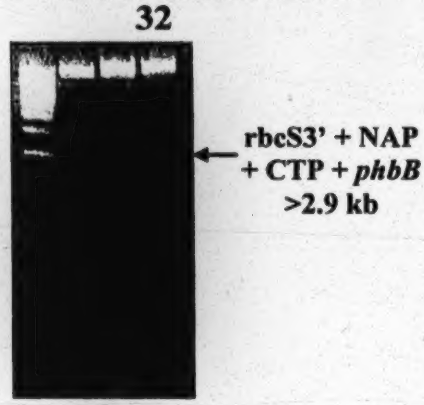
c. Colony PCR of clone 32 for *phbA* amplification

Figure 2-13. Identification of *phbA* and *phbB* genes by pool- and individual colony PCR a.) Pool-colony PCR of 55 colonies, which obtained from transformation of ligation reaction of *phbB* with CTP cassette insert and #16CTP+A:pKYLX-NAP backbone. Ten colonies were used per PCR reaction with *phbB* primers. Number in each lane indicates the pool number. The arrow points at *phbB* positive band of pool #4, which had colonies #31 to #40 as a template. b.) Colony PCR using individual colonies from pool #4 (#31 to #40) as a template. The arrow indicates amplified *phbB* band of clone #32. c.) Colony PCR for *phbA* amplification using clone #32 as a template. The arrow points at positive *phbA* bands in lane #32.

a. Restriction analysis of #32 CTP+A:CTP+B:pKYLX-NAP


32
pKYLX-NAP backbone
rbcS3' + NAP >1.8 kb
CTP + *phbA* 1.6 kb
CTP + *phbB* 1.1 kb

b. Restriction analysis of #32CTP+A:CTP+B: pKYLX-NAP for orientation of *phbB* with CTP cassette insertion


32
rbcS3' + NAP + CTP + *phbB* >2.9 kb

c. Diagrams of two orientations of *phbB* with CTP cassette insertion in #32CTP+A:CTP+B:pKYLX-NAP

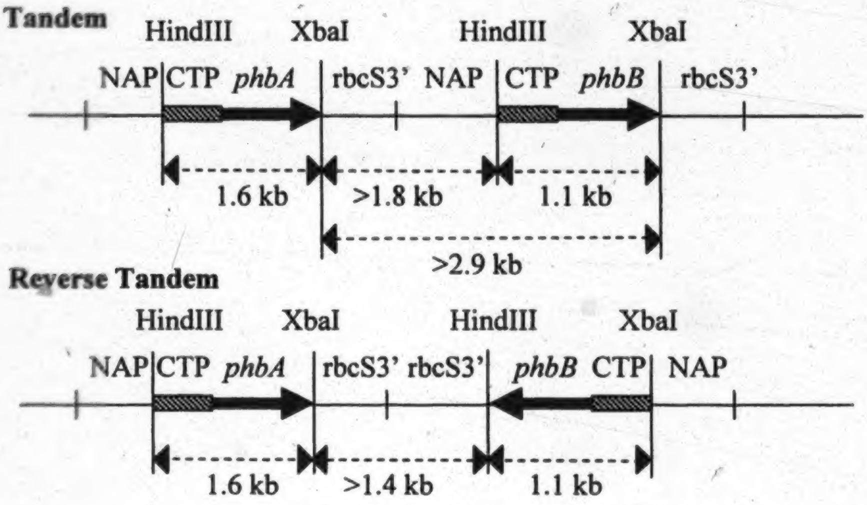

Tandem
HindIII
XbaI
HindIII
XbaI
NAP
CTP
phbA
rbcS3'
NAP
CTP
phbB
rbcS3'
1.6 kb
>1.8 kb
1.1 kb
>2.9 kb
Reverse Tandem
HindIII
XbaI
HindIII
XbaI
NAP
CTP
phbA
rbcS3'
rbcS3'
phbB
CTP
NAP
1.6 kb
>1.4 kb
1.1 kb

Figure 2-14. Restriction analysis of *phbA* and *phbB* genes, and orientation of *phbB* with CTP cassette insertion a.) restriction analysis of #32CTP+A:CTP+B:pKYLX-NAP plasmid with HindIII and XbaI digestion. Arrows indicate bands of target *phbA* and *phbB* with CTP attachments, as well as pKYLX-NAP backbone and rbcS3' with Napin promoter fragment with sizes corresponding to the diagram. b.) Restriction analysis of #32CTP+A:CTP+B:pKYLX-NAP with XbaI to identify orientation of *phbB* cassette. The arrow indicates the digested fragment of 2.9 kb (rbcS3' 0.7 kb, Napin promoter 1.1 kb, CTP 0.4 kb, *phbB* 0.7 kb), which confirmed a tandem order of *phbB* and *phbC* cassettes. c.) Diagrams show restriction sites and sizes of fragments of #32CTP+A:CTP+B: pKYLX-NAP in tandem and reverse tandem orders of *phbA* and *phbB* cassettes.

a size of approximate 1.4 kb (Figure 2-14c). The clone #32 was named #32CTP+A: CTP+B:pKYLX-NAP, re-cultured and stored at -80°C (Figure 2-11e).

2.3.2.4 *phbC* single construct for co-transformations for plastidial expression

pRD320 plasmid is a binary vector carrying *pat* gene for PPT selectable marker within Ti-DNA borders (Figure 2-15a). *pat* encodes phosphinothricin acetyl transferase (PPT), which makes transformed plants resistant to PPT herbicide. *pat* gene cassette, PPT resistance fragment (PPT^R), was utilized in the preparation of *phbC* plastidial constructs. PPT^R fragment was taken from pRD320 plasmid vector by HindIII and EcoRI restriction sites, and ligated into pBluescript SKII(-) by the same sites. The modified vector was named #2PPT:pBSK(-) (Figure 2-15b). Then the PPT^R fragment was taken out of #2PPT:pBSK(-) by ClaI and BamHI restriction sites of the pBluescript SKII(-) backbone, electrophoresed on 1% agarose gel, and gel-purified for insert fragment preparation (Figure 2-16a).

phbC with CTP fragment was taken out of #1CTP+C:pBSK(-) plasmid by HindIII and XbaI restriction sites (Figure 2-6e), and ligated to an empty pKYLX-NAP vector, and transformed into *E. coli* DH5α cells. Both colonies tested by colony PCR and restriction analysis of the plasmids showed presence of *phbC* with CTP (CTP 0.4 kb, *phbC* 1.8 kb). Colony #11 was named #11CTP+C:pKYLX-NAP, re-cultured and stored at -80°C (Figure 2-15d).

Kanamycin resistance fragment (Kan^R) of #11CTP+C:pKYLX-NAP was taken out of the construct by ClaI and BamHI restriction sites (Figure 2-15d). The backbone was gel-purified, ligated with PPT^R fragment previously prepared, and transformed into *E. coli* DH5α host. Restriction analysis with BglII was performed on plasmid isolated from two colonies to confirm the presence of PPT^R fragment (Figure 2-16c). Clone #2 showed presence of correct insert of approximate 2.0 kb (Figure 2-16b). This result confirmed a successful substitution of Kan^R fragment with PPT^R fragment because there is one BglII restriction site in the PPT^R fragment, but none in Kan^R fragment. This clone was named #2PPT:CTP+C:pKYLX-NAP, re-cultured and stored at -80°C (Figure 2-15e).

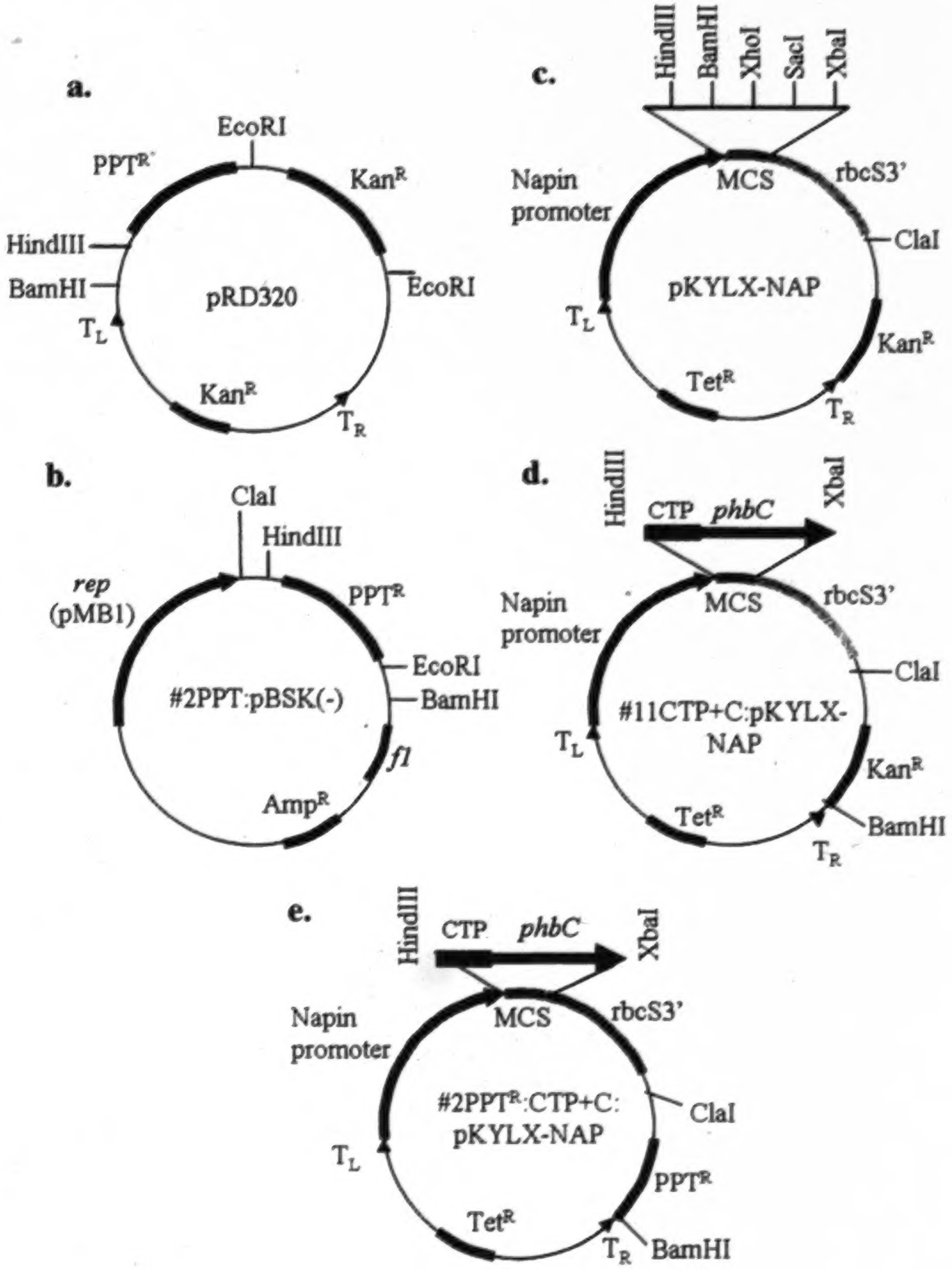
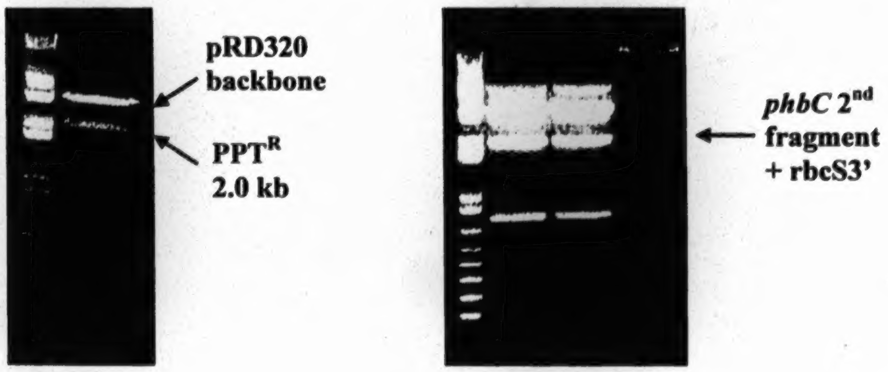

a.
EcoRI
PPT^R
Kan^R
HindIII
BamHI
EcoRI
pRD320
T_L
Kan^R
T_R
b.
ClaI
HindIII
rep
(pMB1)
PPT^R
EcoRI
BamHI
#2PPT:pBSK(-)
f1
Amp^R
c.
HindIII
BamHI
XhoI
SacI
XbaI
Napin
promoter
MCS
rbcS3'
ClaI
pKYLX-NAP
T_L
Kan^R
Tet^R
T_R
d.
HindIII
CTP
phbC
XbaI
Napin
promoter
MCS
rbcS3'
ClaI
#11CTP+C:pKYLX-NAP
T_L
Kan^R
Tet^R
BamHI
T_R
e.
HindIII
CTP
phbC
XbaI
Napin
promoter
MCS
rbcS3'
#2PPT^R:CTP+C:
pKYLX-NAP
ClaI
T_L
PPT^R
Tet^R
T_R
BamHI

Figure 2-15. Preparation of *phbC* single construct for co-transformation for plastidial expression a.) pRD320 binary gene expression vector. The vector contains Kanamycin resistance (Kan^R) and PPT herbicide resistance (PPT^R) markers within left and right boarders (T_L and T_R). There is another Kanamycin resistance marker located outside the boarders. b.) #2PPT:pBSK(-) plasmids contain PPT herbicide resistance fragment (PPT^R) in multiple cloning sites (MCS) of pBluescript SKII(-) vector carrying an Ampicillin resistance fragment (Amp^R). c.) pKYLX-NAP binary gene expression vector. The vector contains a seed-specific Napin promoter, multiple cloning sites (MCS) for gene insertion, rbcS3' terminator, a Kanamycin resistance marker (Kan^R) and a ClaI restriction site between left and right boarders (T_L and T_R). Tetracycline resistance marker is located outside the boarders. d.) #11CTP+C:pKYLX-NAP vector. *phbC* with CTP attachment was inserted into pKYLX-NAP by HindIII and XbaI restriction sites in multiple cloning region. e.) #2PPTR:CTP+C:pKYLX-NAP plasmid. Kanamycin resistance fragment (Kan^R) was taken out of #11CTP+C:pKYLX-NAP and replaced with PPT^R fragment from #2PPT:pBSK(-) by ClaI and BamHI restriction sites.

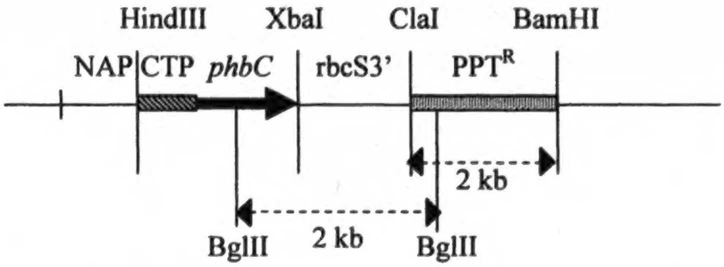


a. Restriction analysis of #2PPT:pBSK(-)

b. Restriction analysis of #2PPT:CTP+C:pKYLX-NAP

pRD320 backbone
PPT^R 2.0 kb
phbC 2nd fragment + rbcS3'

c. Diagram of *phbC* with CTP cassette and PPT resistance fragment of #2PPT:CTP+C:pKYLX-NAP



HindIII
XbaI
ClaI
BamHI
NAP
CTP *phbC*
rbcS3'
PPT^R
2 kb
BglII
2 kb
BglII

Figure 2-16. Restriction analysis of #2PPT:pBSK(-) and #2PPT:CTP+C:pKYLX-NAP, and the diagram of *phbC* cassette with PPT resistance fragment a.) restriction analysis of#2PPT:pBSK(-) plasmid with ClaI and BamHI digestion. Arrows indicate bands of target PPT resistance fragment (PPT^R) and pRD320 backbone. b.) restriction analysis of #2PPT:CTP+C:pKYLX-NAP with BglII digestion. The arrow indicates presence of correct insert of 2.0 kb. c.) Diagram shows restriction sites and sizes of fragments of #2PPT:CTP+C:pKYLX-NAP.

#32CTP+A:CTP+B:pKYLX-NAP and #2PPT^R:CTP+C:pKYLX-NAP were designed to have different selectable markers within Ti-DNA borders, which is convenient for co-transformation into the same plant line. Plants which were successfully transformed with both constructs will be resistant to Kanamycin as well as PPT herbicide, and will have all three *phb* genes.

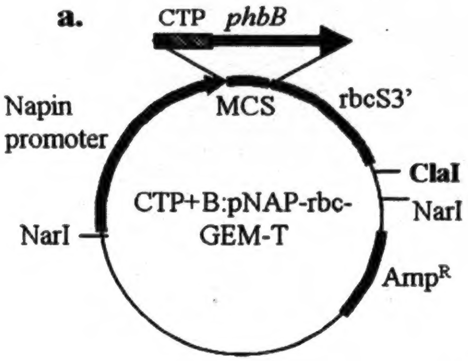
2.4 Future work

In this study two gene constructs were created for co-transformation to introduce PHB synthesis pathway into leucoplasts in developing seeds. These constructs had different selectable markers as discussed in 2.3.2.4. Transformation of both gene constructs into the plants can be done in different approaches. First, plants can be transformed at the same time with both constructs (co-transformation) and screened for T_0 plants that have resistance to both Kanamycin and PPT herbicide. However, the probability of two Ti-DNAs from both gene constructs getting into the same plant is very low. This approach would require a large number of plants to start with, in order to increase the possibility of successful co-transformation. The second approach would be to introduce one Ti-DNA after another. After transformation with one gene construct, transformants from the first transformation would subsequently be used in the second transformation with the other gene construct, and screened for co-transformants with both marker resistances. Ideal conditions would be to use single copy homozygous plants for the second transformation. Thus, transformation procedures must be performed twice, which would be time consuming and would require a large amount of work. The third approach would be to raise separate transformants, select for homozygous lines, and hybridize them to obtain double-transformants.

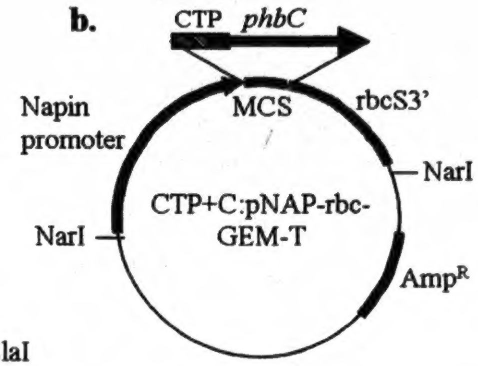
However, the best approach would be to create a triple-cassette construct carrying all three *phb* genes. This could be done using similar strategy as preparation of a double-cassette construct. Another ClaI restriction site could be created between NarI site and *rbcS3'* terminator of pNAP-rbc-GEM-T with *phbB* insert (Figure 2-17a). Meanwhile, another modified pNAP-rbc-GEM-T vector carrying *phbC* with CTP sequence insert could be prepared with the gene cassette flanked by this NarI restriction sites (Figure 2-

17b). After introduction of *phbB* gene cassette into *phbA* construct by using the compatibility of NarI and ClaI cleavage ends, the ligated sites can not be re-cleaved by any of the restriction endonucleases (Figure 2-17c). Thus the third gene cassette (*phbC*) could be inserted into the double cassette construct by the newly engineered ClaI restriction site (Figure 2-17c). This would ultimately result in one triple-cassette construct (Figure 2-17d) carrying every essential element for introduction of PHB synthesis into leucoplast in developing seeds, and could be introduced into plants in one transformation procedure.

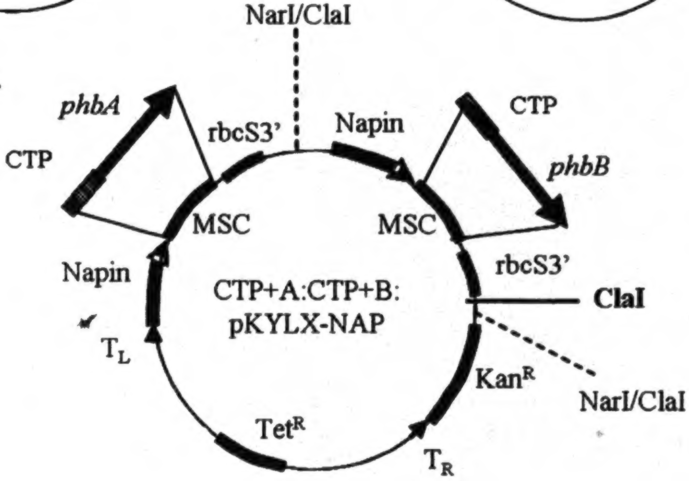
a.


CTP *phbB*
Napin promoter
MCS
rbcS3'
ClaI
NarI
NarI
CTP+B:pNAP-rbc-GEM-T
Amp^R

b.


CTP *phbC*
Napin promoter
MCS
rbcS3'
NarI
NarI
CTP+C:pNAP-rbc-GEM-T
Amp^R

c.


NarI/ClaI
phbA
CTP
rbcS3'
Napin
CTP
phbB
MSC
MSC
Napin
rbcS3'
ClaI
CTP+A:CTP+B:
pKYLX-NAP
T_L
Kan^R
NarI/ClaI
Tet^R
T_R

d.

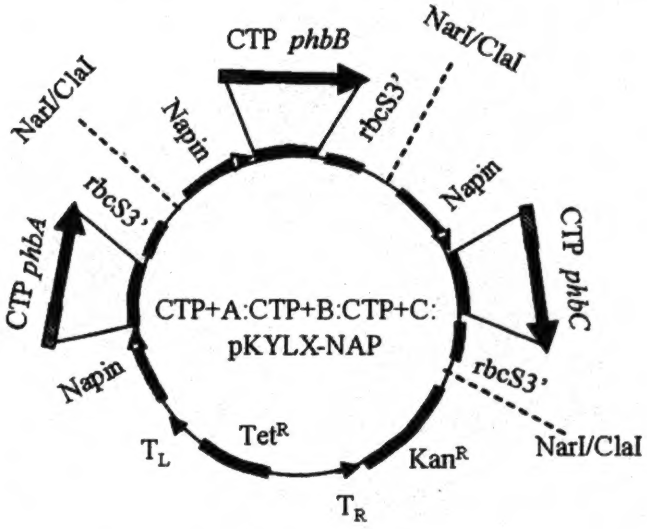

CTP *phbB*
NarI/ClaI
NarI/ClaI
Napin
rbcS3'
rbcS3'
Napin
CTP *phbA*
CTP *phbC*
CTP+A:CTP+B:CTP+C:
pKYLX-NAP
rbcS3'
Napin
NarI/ClaI
T_L
Tet^R
Kan^R
T_R

Figure 2-17. Preparation of triple-cassette construct for plastidial single transformation a.) CTP+B:pNAP-rbc-GEM-T vector containing a ClaI restriction site created between NarI site and rbcS3' terminator. b.) CTP+C:pNAP-rbc-GEM-T vector containing *phbC* with CTP cassette flanked by this NarI restriction sites. c.) CTP+A:CTP+B:pKYLX-NAP vector with *phbB* gene cassette inserted into *phbA* construct by using the compatibility of NarI and ClaI cleavage ends, which can not be re-cleaved by any of the restriction endonucleases. d.) CTP+A:CTP+B:CTP+C:pKYLX-NAP triple construct with *phbC* cassette inserted into the double cassette construct by the newly created ClaI restriction site.

2. 5 References

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Chapter 3 Phb protein expression and purification

3.1 Introduction

3.1.1 Three essential enzymes for PHB synthesis

In a natural producer, *R. eutropha*, PHB is synthesized from an initial substrate, acetyl-CoA, by a sequence of three enzymatic reactions catalyzed by three enzymes: β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase. These three enzymes are encoded by three *phb* genes; *phbA*, *phbB* and *phbC*, respectively (refer to 2.1.1). β-ketothiolase, also known as PhbA protein, condenses two molecules of acetyl-CoA to become acetoacetyl-CoA (Figure 3-1). PhbA was reported to have a tetramer structure with identical subunits of 44 kDa on SDS-PAGE analysis. This enzyme has high substrate specificity, and is active exclusively with acetyl-CoA (Haywood et al. 1988a). Acetoacetyl-CoA reductase, PhbB protein, reduces acetoacetyl-CoA and gives 3-hydroxybutyryl-CoA as a product. This enzyme was reported to be NADPH dependent, and has a tetramer form with identical subunits of 25 kDa on SDS-PAGE analysis (Haywood et al. 1988b). PHB synthase, PhbC protein, was reported with a molecular weight of 63 kDa on a SDS-PAGE analysis. PhbC has an important role in polymerization of the monomers. PHB synthase is active with both 3-hydroxybutyryl-CoA (HB) and 3-hydroxyvaleryl-CoA (HV). Therefore PHB synthase from *R. eutropha* has a potential to polymerize both PHB and co-polymer PHBHV when the substrates permit (Haywood et al. 1989). The experiment discussed in this chapter focused on production of recombinant Phb proteins in *E. coli* host for immunization to produce polyclonal antibodies. These antibodies will be used in Western hybridization experiments to investigate Phb protein expressions in transgenic plants.

3.1.2 Over-expression of recombinant proteins

Protein expression vectors used in this study, pET30a-c(+) series (Novagen, Mississauga, ON), belong to the pET system that was developed specifically for cloning and expressing recombinant proteins in *E. coli* host (Figure 3-2a). The target gene is inserted into the plasmid in the multiple cloning region. The expression is controlled by strong bacteriophage T7 transcription signals, which can be dramatically regulated by

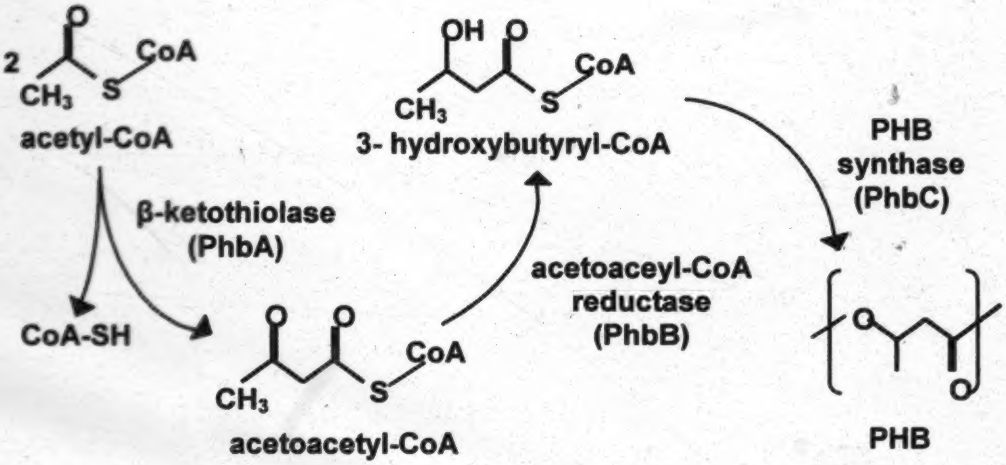

2
CH₃
S
CoA
acetyl-CoA
β-ketothiolase
(PhbA)
CoA-SH
CH₃
S
CoA
acetoacetyl-CoA
acetoaceyl-CoA
reductase
(PhbB)
OH
CH₃
S
CoA
3- hydroxybutyryl-CoA
PHB
synthase
(PhbC)
PHB

Figure 3-1. Three enzymatic reactions for PHB synthesis
β-ketothiolase (PhbA) condenses two molecules of acetyl-CoA to become acetoacetyl-CoA. Acetoacetyl-CoA reductase (PhbB) reduces acetoacetyl-CoA and gives 3-hydroxybutyryl-CoA (HB) monomer as a product. PHB synthase (PhbC) polymerizes the HB monomers to form PHB polymer.

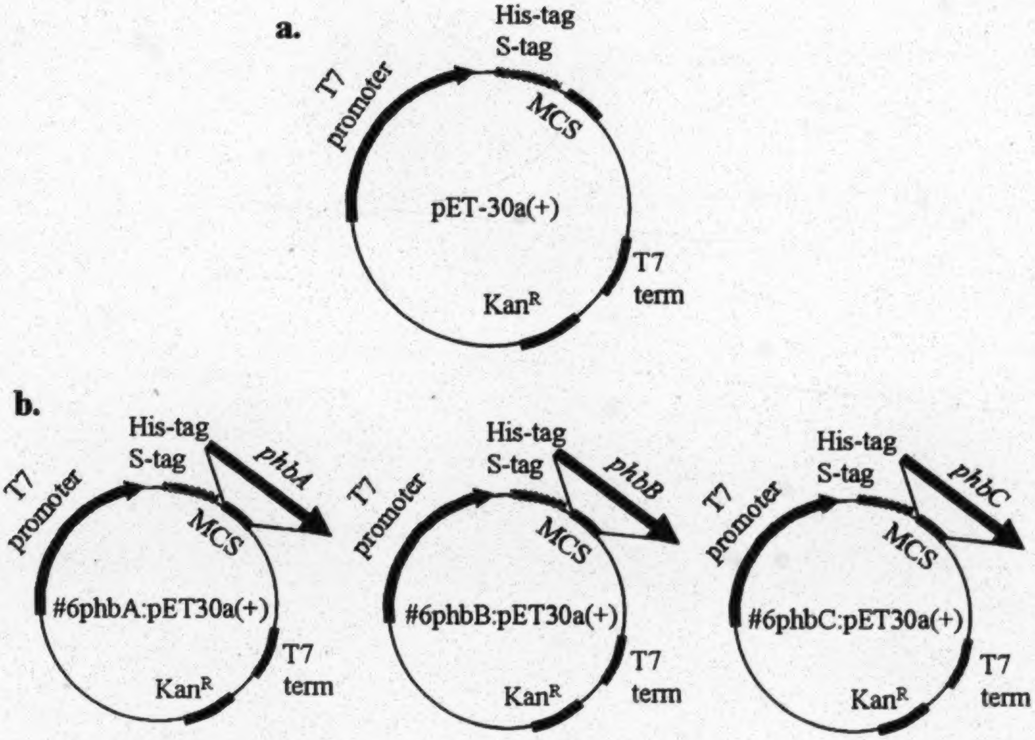

a.
His-tag
S-tag
T7 promoter
MCS
pET-30a(+)
T7 term
Kan^R
b.
His-tag
S-tag
phbA
T7 promoter
MCS
#6phbA:pET30a(+)
T7 term
Kan^R
His-tag
S-tag
phbB
T7 promoter
MCS
#6phbB:pET30a(+)
T7 term
Kan^R
His-tag
S-tag
phbC
T7 promoter
MCS
#6phbC:pET30a(+)
T7 term
Kan^R

Figure 3-2. *phb* genes in protein expression vector
c.) pET-30a(+), protein expression vector with Kanamycin resistance (Kan^R). The vector contains T7 promoter and T7 terminator, His-tag and S-tag sequences upstream of the multiple cloning sites (MCS). b.) #6phbA:pET30a(+), #6phbB:pET30a(+), and #6phbC:pET30a(+) plasmids contain *phbA, phbB* and *phbC*, respectively. The *phb* genes were inserted in multiple cloning sites (MCS) in frame with His-tag and S-tag sequences of pET30a(+) vector.

providing a source of T7 RNA polymerase induction, isopropyl-β-D-thiogalatopyranoside (IPTG), to the host cell. IPTG is a very powerful inducing factor that after fully induced for a few hours, more than 50% of the total cell protein becomes the target protein.

The *E. coli* host strain used in this study, BL21(DE3), is the most extensively used host for recombinant gene expression. BL21(DE3) has a deficiency in *lon* protease and lacks *omp*^{T} outer membrane protease, which can degrade recombinant proteins during downstream purification step (Geodberg and Dunn 1988). Therefore the target proteins produced in the BL21(DE3) host strain were expected to be more stable than those in other stains with normal levels of proteases. It also contains a chromosomal copy of *lacUV5* promoter, *lacI* gene and the gene for T7 polymerase as derivatives of bacteriophage DE3. The transcription of the T7 RNA polymerase gene is exclusively controlled by *lacUV5* promoter, which is inducible by IPTG. Addition of IPTG to the growing culture induces T7 RNA polymerase production, which subsequently induces the transcription of the target DNA in the plasmid. Meanwhile *lacI* gene, which produces *lac* repressor, restricts T7 RNA polymerase activity in the host while the transcription is not induced (Figure 3-3).

3.1.3 His-tag fusion protein

His-tag is a very useful fusion protein for purification of the recombinant protein. Attachment of the DNA sequence coding for 6 Histidine amino acids in frame with the target DNA can facilitate detection of the target protein on Western blots and purification of the target protein by column purification. These His-tag peptides are small in size; nevertheless they are very specific and sensitive to the detection reagents. His-tag is exceptionally useful for target proteins that form inclusion bodies, because it can help increase the solubility and facilitate the purification. His-tagged proteins are purified by passing the cell lysate, which contains total cell proteins, through a nickel column (Ni-column). The resin inside the Ni-column chelates with the His-tag peptide, which results in strong interaction between target protein and the resin inside the column. After other bacterial proteins from the cell lysate are washed off, the target protein can be eluted by adding imidazole into the column.

3.1.4 Summary

Isolated *phb* genes were inserted individually into a protein expression vector downstream of the 6xHistidine tag sequence. The modified vectors were sequenced to confirm a correct reading frame. The three Phb protein expression constructs were transformed individually into expression host, *E. coli* BL21(DE3). Expressions of Phb proteins were induced by addition of IPTG to cell culture. Size, solubility, and expression level of each protein were analyzed using SDS-PAGE and immunodetection with HRP-conjugated anti-His antibodies (Western blot). The production of recombinant proteins was increased by scaling up the amount of cell culture, and the length of culture time. Target proteins were extracted and purified using Ni-NTA fast start kit (Qiagen). Purified protein solutions were quantified following a modified Bradford assay. The Phb protein samples were finally sent to University of Calgary SACRI Antibody Services for production of polyclonal antibodies.

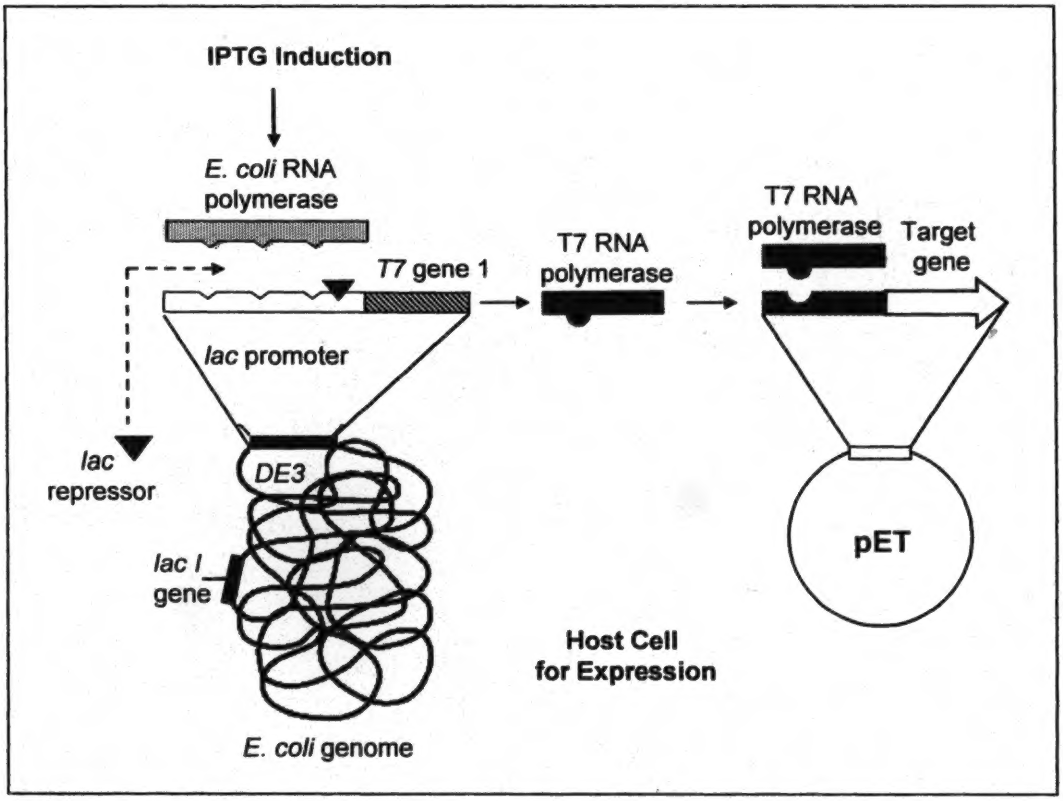

IPTG Induction
E. coli RNA polymerase
T7 gene 1
lac promoter
T7 RNA polymerase
T7 RNA polymerase
Target gene
lac repressor
DE3
lac I gene
pET
Host Cell for Expression
E. coli genome

Figure 3-3. *E. coli* BL21(DE3) protein expression host
BL21(DE3) carries *lac* promoter, *lacI* gene and the gene for T7 polymerase on its chromosome. The transcription of the T7 RNA polymerase gene is controlled by *lac* promoter, which is inducible by IPTG. IPTG induces T7 RNA polymerase production, which subsequently induced the transcription of the target DNA in the plasmid. *lacI* gene, which produces *lac* repressor, provides a basal T7 RNA polymerase activity in the host while the transcription is not induced. (Figure from pET system manual 11th edition, page 49)

3.2 Materials and Methods

3.2.1 Protein expression vector, pET30a(+), and expression host strain

pET30a(+) vector belongs to the pET30a-c(+) vector series (Novagen), which provides three separate reading frames to facilitate expression of recombinant proteins in *E. coli* host. pET30a(+) is a 5.4 kb plasmid; consisting of 6xHistidine tags at both N- and C-termini enclosing multiple cloning region, and Kanamycin selectable marker gene cassette (Figure 3-2). Target *phb* genes were sub-cloned into pET30a(+) plasmid under control of bacteriophage T7 transcription. The target genes in pET30a(+) were initially introduced into non-expression host, DH5α, which does not contain T7 RNA polymerase gene. Once the presence and sequence of the target genes were confirmed, the plasmid was transferred into BL21(DE3) expression host, with a chromosomal copy of T7 RNA polymerase gene under *lacUV5* promoter. *lacUV5* is a promoter inducible by IPTG. Therefore the expression of the target gene can be induced by supplementing IPTG to the expression host culture. Both DH5α and BL21(DE3) were grown in LB liquid media and LB plates at 37°C with appropriate antibiotics.

3.2.2 Transformation of competent *E. coli* BL2(DE3) cells

1.75 μL of plasmid DNA carrying protein expression cassette, extracted using Miniprep Kit (Qiagen), was mixed with a 20 μL aliquot of competent *E. coli* BL21(DE3) cells (Novagen), previously thawed on ice. The mixture was incubated on ice for 15 min, followed by a heat shock at 42°C for 45 sec, and a cold shock on ice for 4 min. 300 μL of pre-warmed SOC liquid media at 37°C was added to the cell mixture, and incubated at 37°C for 1 hour. The cell suspension was plated (200 μL/plate) on LB plates with Kanamycin 50 μg/mL.

3.2.3 Inductions of recombinant proteins (pilot expression)

1 mL of culture grown overnight at 37°C was added into 19 mL of fresh liquid LB media (Kanamycin 50 μg/mL) and incubated with shaking at 37°C for 3 hours, or until OD_{600} reached approximately 0.6. 500 μL aliquot of the culture was collected before induction (Time point = 0). IPTG was added to the cell culture to a final concentration of 1 mM. The culture was incubated at 37°C with shaking for 4 hours. Samples of the

culture were collected every hour post-induction (Time point = 1, 2, 3, and 4). Bacterial cells from all samples were collected by centrifugation at 14,000 rpm for 2 min, at RT and kept frozen at -20°C overnight or until further use.

3.2.4 Preparation of protein samples (pilot expression) and SDS-PAGE

Frozen cell pellets collected from liquid culture prior- and post-induction were thawed on ice for 20 min. Pellet samples were treated with the following buffers and enzymes: 118 µL phosphate-buffered saline (PBS) (10 mM, pH 7.4), 1.5 µL lysozyme (100 mg/mL), 0.75 µL dithiothreitol (DTT) (1 M), and 1.5 µL phenylmethylsulphonyl fluoride (PMSF) (100 mM). After vigorous shaking (or vortex), the pellet samples were incubated on ice for 30 min. 7.5 µL DNase I (100 µg/mL), 7.5 µL RNase I (100 µg/mL) and 15 µL Triton-X 10% (v/v) were added, followed by vortex and incubation on ice for 15 min. To test solubility of the protein, soluble and insoluble fractions of samples collected 4 hours post-induction (T = 4) were prepared by sonication for 1-2 min to obtain cell lysate and centrifugation at 14,000 rpm for 2 min, RT. Supernatant (soluble fraction) was transferred to a fresh tube, while the pellet (insoluble fraction) was resuspended in 100 µL PBS (10 mM, pH 7.4). 25 µL 2xSDS sample buffer was added to 25 µL of each sample and the cell debris suspension. Denaturation of the protein samples was done by heat at 90°C for 5 min. After cooling down to RT, 25 µL of each sample was subsequently loaded onto SDS-PAGE gel (5% stacking gel, and 12% resolving gel), and electrophoresed at 160 voltage (Mini-PROTEAN 3 Cell, BIO-RAD, Ottawa, ON). The gels were stained with 0.05% (w/v) coomassie blue in methanol(5v): acetic acid(1v): water(4v) solution for 1hour, and destained overnight with 5% methanol, 7% acetic acid in water.

3.2.5 Immunodetection of His-tagged proteins

SDS-PAGE gel containing protein samples to be examined was equilibrated in transfer buffer (48 mM Tris base, 39 mM Glycine, 1.3 mM SDS, pH 9.2) for 25 min at RT. Polyvinylidene Difluoride membrane (PVDF) was cut according to the size of the gel, soaked with pure methanol, and equilibrated in transfer buffer for 25 min at RT.

Separated proteins on SDS-PAGE gel were transferred and immobilized onto the PVDF membrane using semi-dry western apparatus (BIO-RAD) applying 15 voltage for 25 min. Immunodetection (western blot) with horseradish peroxidase (HRP) conjugated anti-His antibodies were performed using QIA*express* Detection and Assay Kit (Qiagen) according to the protocol supplied by the manufacturer. Transferred membrane was washed twice with Tris Buffered Saline buffer (TBS buffer, 50 mM Tris Base, NaCl 0.09% (w/v), pH 7.6) for 10 min at RT, and incubated overnight in blocking solution (Blocking reagent 0.5% (w/v), Tween20 0.1% (v/v) in 1x Blocking reagent buffer, Qiagen) at 4°C. The membrane was washed twice with TBS-T buffer (Tween20 0.1% (v/v) in TBS buffer) and once with TBS buffer for 10 min at RT. After the incubation with HRP conjugated anti-His antibodies (1/1000 dilution in blocking solution supplied, Qiagen) for 1 hour at RT, the membrane was washed with TBS-T buffer to remover unbound antibodies, and again with TBS buffer. Anti-His conjugated bands were visualized by using TMB (tetramethylbenzidine) substrate (Vector Laboratories Inc., Burlington, ON). The membrane was incubated in staining solution for 5-10 min at RT. The reaction was stopped by washing the membrane twice with distilled water and air-dried.

3.2.6 Scale up expression of recombinant proteins

Following the pilot expression, the quantity of cell culture was scaled-up to prepare sufficient production of recombinant proteins for purification and further use. 250 mL of LB media (Kanamycin 50μg/mL) was inoculated with 10 mL overnight grown culture, and incubated at 37°C with vigorous shaking until OD_{600} reached to 0.6. IPTG was added to the culture to a final concentration of 1 mM to induce expression of the target gene. The culture was incubated for additional 4 hours. Non-induced control and induced control samples were collected from 500 μL of the culture prior and post induction, and prepared as described in 2.2.5. The cells were harvested from the culture by centrifugation at 4,000 x g for 20 min at RT. Cell pellets were stored overnight at -20°C.

3.2.7 Purification of recombinant proteins

Purifications of 6xHistidine-tagged proteins were performed under both native and denaturing conditions using Ni-NTA Fast Start Kit (Qiagen). In the native condition protocol, cell pellets were lysed in the lysis buffer (pH 8.0) to obtain cell lysate. The cell lysate was then centrifuged at 14,000 x g for 30 min at RT to separate cell debris from supernatant. The supernatant was passed through Ni-NTA 6xHistidine-tag binding column, followed by washing twice with wash buffer (pH 8.0). The purified proteins were eluted in elution buffer (pH 8.0) containing imidazole. In the denaturing condition, cell pellets were resuspended in denaturing lysis buffer (pH 8.0), and incubated at RT for 1 hour. The lysate was then centrifuged at 14,000 x g for 30 min at RT, to obtain the supernatant with target proteins. The supernatant was passed through the Ni-NTA column, followed by washing twice with denaturing wash buffer (pH 6.3). The purified target proteins were eluted in denaturing elution buffer (pH 4.5) containing imidazole. Sample fractions were taken from flow through of each step. The efficiency of purification, yield and purity of the purified recombinant proteins were analyzed by SDS-PAGE.

3.2.8 Quantification of purified proteins

Extracted and purified protein samples were quantified using a modified Bradford assay with BSA (bovine serum albumin) as the standard. Two duplicate sets of the BSA protein standards of 0 μg, 2 μg, 5 μg, 10 μg, and 15 μg were prepared in rehydration buffer (Bio-Rad) to a final volume of 20 μL. The protein samples were diluted into 1:10 and 1:5 to a final volume of 20 μL in the elution buffer as used to elute protein from purification column. 80 μL of 0.12 N HCL was added to each of the protein standards and samples, followed by 1 mL of 1:4 diluted protein assay dye (Bio-Rad). The solutions were incubated at RT for 5 minutes. 200 μL of each protein standards and samples was transferred into a well of microtiter plate. The absorbance values of the proteins solutions was measured at 595 nm from the two duplicates by a microplate reader. The peak absorbance, which corresponds to the amount of protein present in the sample, was determined by the binding of coomassie blue in protein assay dye solution to the side chains of tyrosine amino acids (Bradford 1976). Average absorbance values obtained from known BSA standards were used to draw a standard curve, which was subsequently used to calculate the concentrations of the protein samples.

3.2.9 Immunization to produce polyclonal antibodies

After purity and quantity of protein samples obtained from the purification step were determined. 2.0 mg of each purified protein samples was sent University of Calgary SACRI Antibody Services for immunizing 2 rabbits, to produce polyclonal antibodies. The elution buffer provided in the Ni-NTA Fast Start Kit (Qiagen) had the conditions already adjusted to meet the injectable criteria for immunization.

3.3 Results and Discussion

3.3.1 Preparation of Phb protein expression vectors

phb genes, which encode three enzymes critical for PHB synthesis, were taken out from #4phbA:pBSK(-), #1phbB:pBSK(-), and #2phbc:pBSK(-) plasmids by EcoRI and XbaI restriction sites (Refer to 2.3.1). The fragments were gel-purified and ligated individually into blank pUC19 cloning vector by the same restriction sites. Plasmids were isolated from white colonies obtained from blue-white selections with X-gal on LB agar plates containing Ampicillin (100 μg/mL). These newly modified vectors were named phbA:pUC19, phbB:pUC19 and phbC:pUC19 respectively. Presence of an insert gene was confirmed by restriction analysis with EcoRI and HindIII digestion. Digested products showed expected bands with sizes of *phbA* 1.2 kb, *phbB* 0.7 kb and *phbC* 1.8 kb. The three target genes were then taken out from modified pUC19 vectors with EcoRI and HindIII restriction sites, and ligated separately into the protein expression vector, pET30a(+) (Novagen), which was previously digested with the same enzymes. Transformed *E. coli* DH5α cells were grown on LB plates containing Kanamycin (50 μg/mL) as a selectable marker. Insertion of each *phb* gene was confirmed by colony PCR of 6 colonies. PCR reaction was performed as previously described. All 6 colonies from each transformation showed the presence of *phb* gene insertion.

Plasmid DNA was extracted from one of these six colonies and named #6phbA:pET30a(+), #6phbB:pET30a(+) and #6phbC:pET30a(+) (Figure 3-2b). The correct reading frame of the *phb* gene with its N-terminal His-tag sequence was confirmed by sequencing the plasmid DNA using T7 promoter and T7 terminator sequencing primers and the internal primers designed for each insert gene. The vectors were subsequently transformed into a protein expression host, *E. coli* BL21(DE3) (Novagen), and plated on LB plates with Kanamycin selection. Colony PCR confirmed the presence of the correct plasmid in the colonies. Six colonies randomly selected from each transformation all showed the correct size *phb* bands (Figure 3-4). Colony #4 with *phbA* insert was selected and named #4phbA:pET30a(+) BL21(DE3), so were #4 phbB:pET30a(+) BL21(DE3) and #2phbC:pET30a(+) BL21(DE3). Because the stop codon of the *phb* genes were kept intact throughout sub-cloning, His-tag was produced only in N-terminus of the recombinant proteins.

3.3.2 Expression levels and solubility of recombinant proteins

Expression levels and solubility of recombinant Phb proteins in modified protein expression vectors, pET30a(+), were investigated in small scale production of 20 mL culture. Productions of target proteins were induced by adding IPTG to the liquid culture to a final concentration of 1 mM. Cell pellets were collected from 500 µL aliquots of the culture at time point = 0, 1, 2, 3 and 4 hours by centrifugation (refer to 3.2.3). The pellet samples were treated as described in 3.2.4 to obtain total bacterial soluble proteins including the induced recombinant proteins. The protein samples were loaded onto SDS-PAGE gels, electrophoresed, and stained with coomassie blue to inspect expression levels at each time point as well as solubility of the recombinant proteins (Figure 3-5).

Non-transformed BL21(DE3) host strain was used as a negative control in this experiment. As shown in figure 3-5a, protein samples in lanes BL T_0 and BL T_3 were prepared from the negative control culture before and 3 hours after induction, respectively. As expected, this result showed that induction with IPTG did not regulate expressions of native bacterial proteins, and confirmed the absence of target Phb proteins in non-transformed bacterial lysate of BL21(DE3) expression host (Figure 3-5a).

SDS-PAGE gel of recombinant PhbA protein showed target bands with an appropriate size (PhbA 44 kDa with attachment of N-terminal His-tag and S-tag). To optimize visualization of the target bands, Western blot analysis was performed utilizing immunodetection by HRP conjugated anti-His antibodies, which bind to His-tag region of the recombinant protein. Protein samples run on the SDS-PAGE gel were subsequently transferred and immobilized onto a PVDF membrane using semi-dry Western apparatus (BIO-RAD). After hybridization with HRP conjugated anti-His antibodies, the target bands were visualized by reacting with TMB (tetramethylbenzidine) substrate (Vector Laboratories) (Figure 3-6a). Western blot results confirmed expression level and solubility of PhbA protein. The absence of bands in BL T_0 and BL T_3 indicated no expression of His-tagged proteins in the negative control even after 3 hours post induction. A small band in T_0, time point = T0, showed a basal expression of PhbA protein prior to induction. The intensity of the target PhbA band increased gradually with induction time after the addition of IPTG (lane T_1 to T_4, correspondent to T = 1, 2, 3 and 4 hours). The last two lanes were supernatant and a cellular debris pellet prepared after cell-lysis from protein samples collected 4 hours post-induction. The intense band from the soluble fraction confirmed the solubility of PhbA as reported earlier (Haywood et al. 1988a). A small band from the insoluble fraction suggested that some target protein was left behind in the insoluble fraction possibly due to incomplete cell-lysis. SDS-PAGE gel of PhbB protein showed target bands slightly above 25 kDa compared to the marker (PhbB 25 kDa with addition of N-terminal His-tag and S-tag) (Figure 3-5b). Proteins separated on the gel were transferred to PVDF membrane to perform an immunodetection with HRP conjugated anti-His antibodies as described earlier. A band in lane T_0 showed a basal expression of PhbB protein at T = 0. Unlike PhbA, the expression level of PhbB did not show a continuous increase with post-induction time. The bands appeared in lanes T_1 to T_4, which represent protein samples collected at T = 1, 2, 3, and 4 hours post induction, showed slight or no changes in intensity. Nevertheless, they appeared moderately larger than the basal expression band (Figure 3-6b). This result indicated that PhbB protein was immediately induced within the first hour after IPTG addition, and the expression level

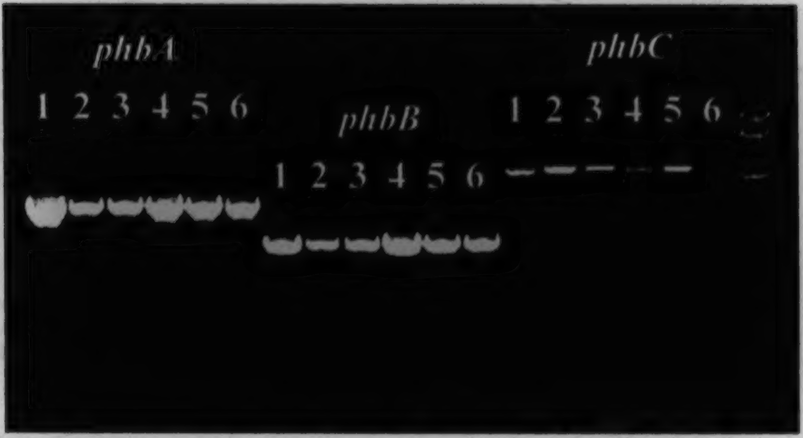

phbA
1 2 3 4 5 6
phbB
1 2 3 4 5 6
phbC
1 2 3 4 5 6

Figure 3-4. Colony PCR for *phb* gene amplifications
Colony PCR using 6 colonies from #6phbA:pET30a(+), #6phbB:pET30a(+), and #6phbC:pET30a(+) transformation into BL21(DE3) host. Number in each lane indicates the colony number. All of the amplified bands showed the correct size products (*phbA* 1.2 kb, *phbB* 0.7 kb, *phbC* 1.8 kb)

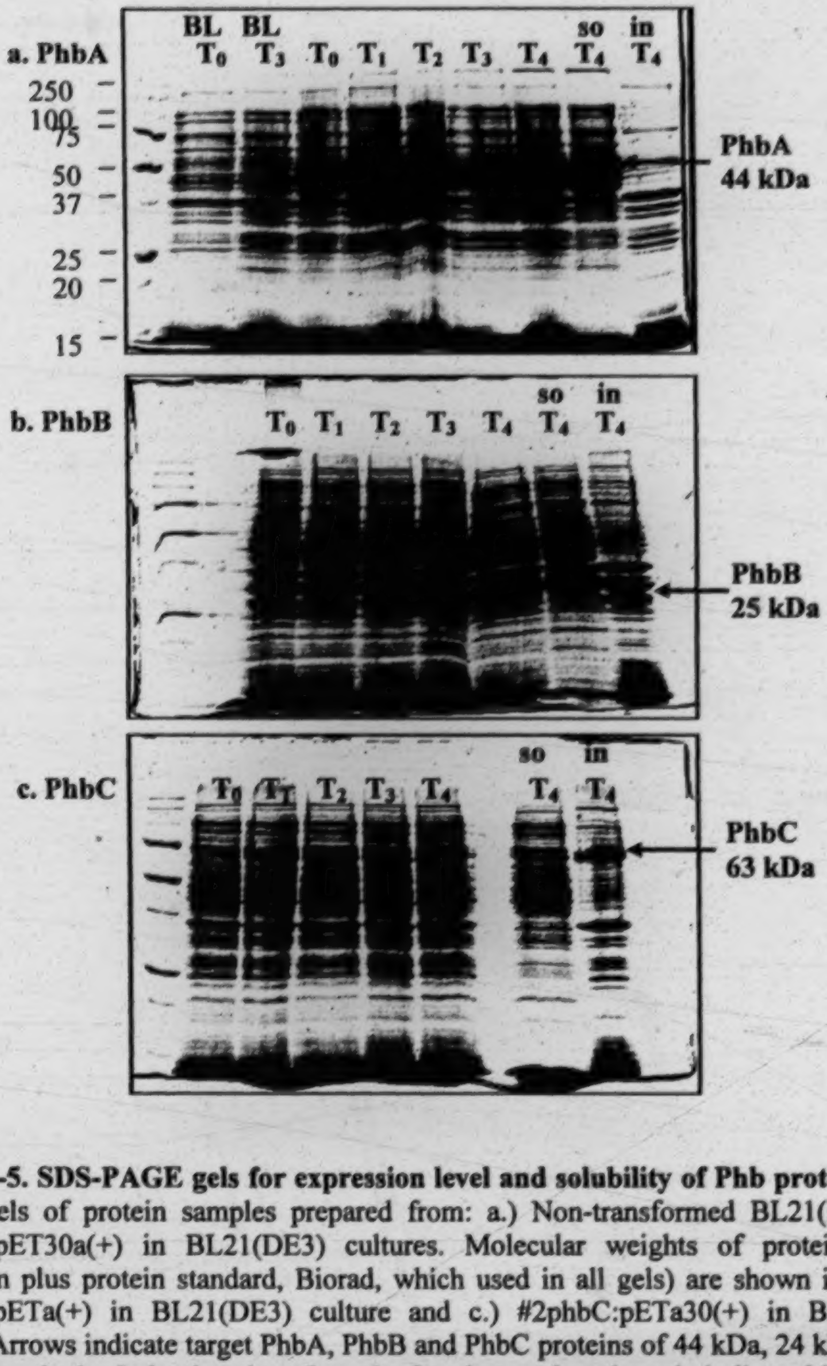

a. PhbA
BL T0
BL T3
T0
T1
T2
T3
T4
so T4
in T4
250
100
75
50
37
25
20
15
PhbA 44 kDa
b. PhbB
T0
T1
T2
T3
T4
so T4
in T4
PhbB 25 kDa
c. PhbC
T0
T1
T2
T3
T4
so T4
in T4
PhbC 63 kDa

Figure 3-5. SDS-PAGE gels for expression level and solubility of Phb proteins SDS-PAGE gels of protein samples prepared from: a.) Non-transformed BL21(DE3) and #4phbA:pET30a(+) in BL21(DE3) cultures. Molecular weights of protein marker (Precision plus protein standard, Biorad, which used in all gels) are shown in kDa b.) #4phbB:pETa(+) in BL21(DE3) culture and c.) #2phbC:pETa30(+) in BL21(DE3) culture. Arrows indicate target PhbA, PhbB and PhbC proteins of 44 kDa, 24 kDa and 63 kDa, respectively. Induction time (hours) of each sample culture is indicated by number on top of each lane ($T_0 - T_4$). Soluble fraction (so) and insoluble fraction (in) of T_4 sample culture are shown in the last two lanes of each gel.

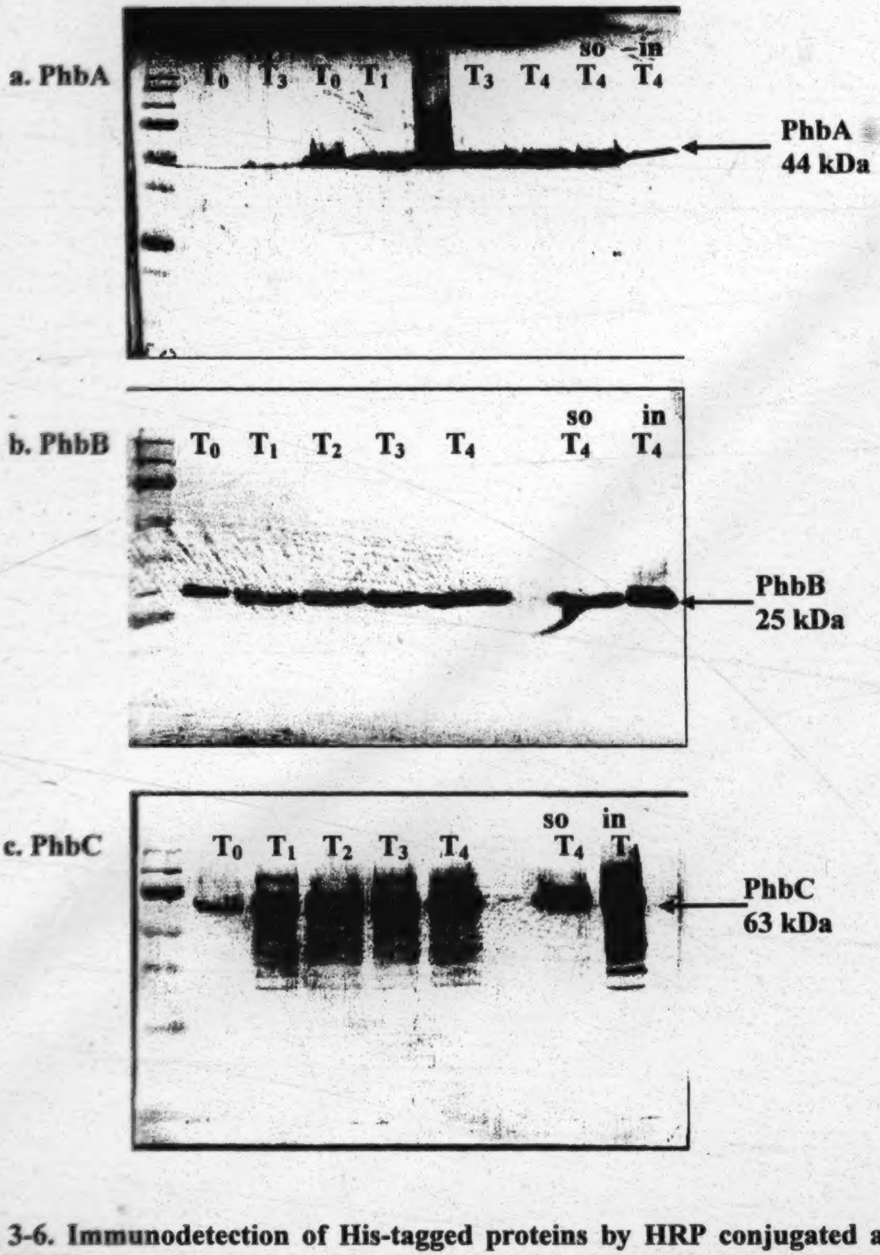

a. PhbA
T_0 T_3 T_0 T_1 T_3 T_4 so T_4 in T_4
PhbA 44 kDa
b. PhbB
T_0 T_1 T_2 T_3 T_4 so T_4 in T_4
PhbB 25 kDa
c. PhbC
T_0 T_1 T_2 T_3 T_4 so T_4 in T_4
PhbC 63 kDa

Figure 3-6. Immunodetection of His-tagged proteins by HRP conjugated anti-His antibodies Western blot analysis of: a.) PhbA SDS-PAGE gel, b.) PhbB SDS-PAGE gel and c.) PhbC SDS-PAGE gel. Arrows indicate detected PhbA, PhbB and PhbC proteins of 44 kDa, 24 kDa and 63 kDa, respectively. Induction time (hours) of each sample culture is indicated by number on top of each lane ($T_0 - T_4$). Soluble fraction (so) and insoluble fraction (in) of T_4 sample culture are shown in the last two lanes of each membrane.

was maintained up to at least 4 hours. Bands in the last two lanes represent supernatant and cellular debris pellet of the protein samples collected 4 hours post-induction. Target bands were found in both soluble and insoluble fractions with similar intensity and size, which might be due to the incomplete cell-lysis. PhbB was earlier reported to be soluble protein (Haywood et al. 1988b).

SDS-PAGE gel of PhbC protein showed target bands of 63 kDa with addition of N-terminal His-tag and S-tag (Figure 3-5c). Western blot detected a band in lane T_0, which represents basal PhbC protein production prior to induction. Intense bands in lanes T_1 to T_4 represent expression of PhbC protein at 1, 2, 3, and 4 hours post-induction, indicated an increase in expression level after induction. However, the expression level was increased and stabilized within the first hour of induction (Figure 3-6c). PhbC protein was found in both soluble and insoluble fractions of the samples after cell-lysis, shown in lanes T_4 soluble and T_4 insoluble, respectively. As for more intense bands in the insoluble fraction, PhbC protein seemed to remain in the cellular debris pellet more than in the supernatant even after a few trials of cell-lysis with longer sonication time and higher concentration of lysozyme. PhbC was reported to be granule-associated under certain conditions. In carbon limited culture PhbC is predominantly soluble. It becomes granule-associated and forms inclusion bodies, which get attached to the cell membrane under certain point of saturation (Haywood et al. 1989). Therefore to lyse the inclusion bodies of PhbC protein into soluble phase is expected to be more challenging than those of PhbA and PhbB.

3.3.3 Purifications of Phb proteins

Following the investigations of expression levels and solubility of Phb proteins, the volume of cell-culture was scaled up to increase the production of recombinant proteins (refer to 3.2.6). Bacterial cells were collected by centrifugation of 250 mL cell culture at 4 hours post-induction. Cell pellets were treated as described in 3.2.7 under native or denaturing conditions to obtain cell lysates. The lysates were passed through Ni-NTA column (Qiagen), washed twice with wash buffer, and eluted in elution buffer containing imidazole. Sample fractions were taken from each steps of the flow-through solution, and analyzed by SDS-PAGE gel electrophoresis (Figure 3-7 and 3-8). Expression level of the target PhbA at 4 hours post-induction in 250 mL culture (lane 2) showed a significant increase compared to basal expression of PhbA prior to induction (lane1) and only the target protein was induced (Figure 3-7a). The target band showed an appropriate size of 44 kDa. Cell lysate (lane 3), which was obtained by cell lysis under native conditions, showed an intense PhbA band. The flow-through fraction of the lysate (lane 4) showed that bacterial proteins, as well as the target PhbA protein, flowed through the column. Loss of the target protein through the column might be caused by a large amount of His-tagged PhbA, which overwhelmed the binding capacity of the column. Fractions of first and second wash buffer (lanes 5 and 6) showed that contaminants and a small portion of PhbA were washed off the column. A large and intense band in lane 7 showed a purified and concentrated PhbA from the first elution. The second elution (lane 8) had a much smaller band of purified PhbA indicating that most of the target protein was eluted in the first elution. Last elution (not shown) revealed no band confirming that the elution was complete.

Similar to PhbA purification, PhbB was effectively purified under native conditions. Expression level of PhbB at 4 hours post-induction after scaling up to 250 mL culture was shown in lane 2 (Figure 3-7b). The target band of the size slightly above 25 kDa indicated the increase of PhbB production compared to un-induced band on lane 1 and bands of native bacterial proteins at the same time point. Fraction of supernatant from the cell lysis (lane 3) showed more intense bands of total proteins including PhbB, which suggested that bacterial cells were efficiently lysed. Fractions of flow-through of the cell lysate and 2 washes (lanes 4 to 6) indicated that contaminants were completely washed off the column. A large and intense PhbB band in the first elution in lane 7 represented purified and concentrated PhbB, which mainly came off in the first elution. A faint small band in the second elution (lane 8) showed the remaining PhbB in the column, and fraction of the third elution (not shown) with no protein bands confirmed the complete elution of the target protein in the first and second elutions.

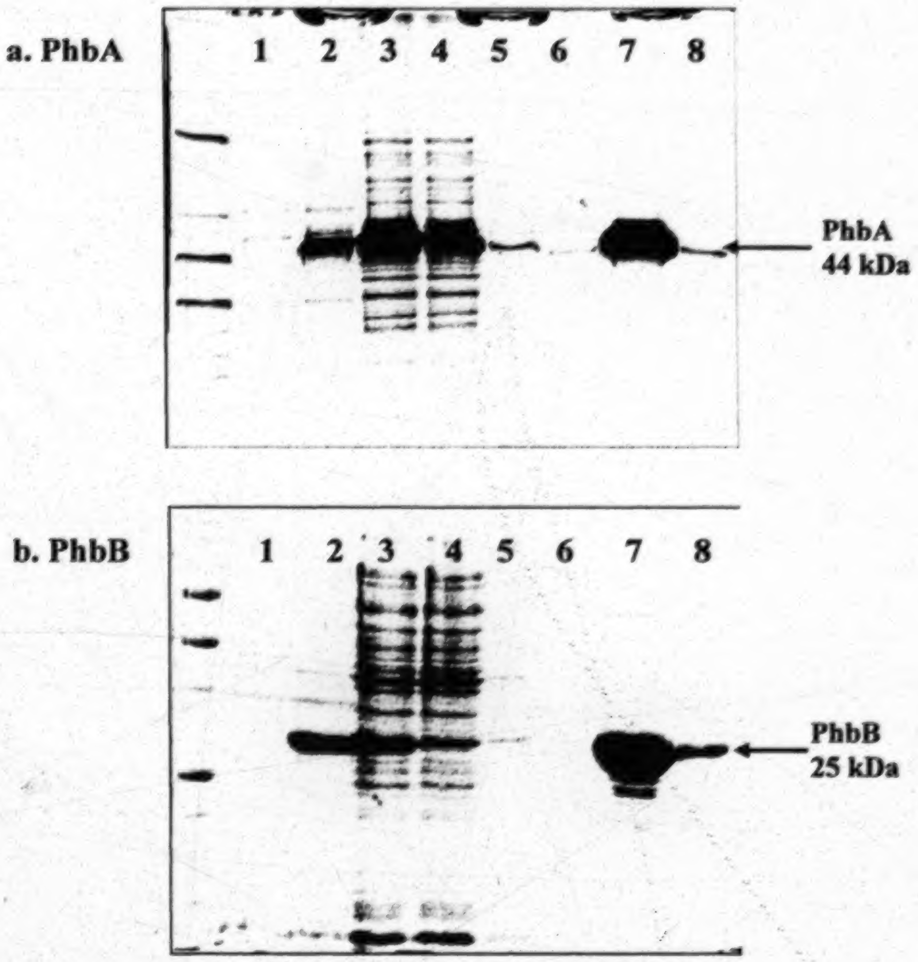
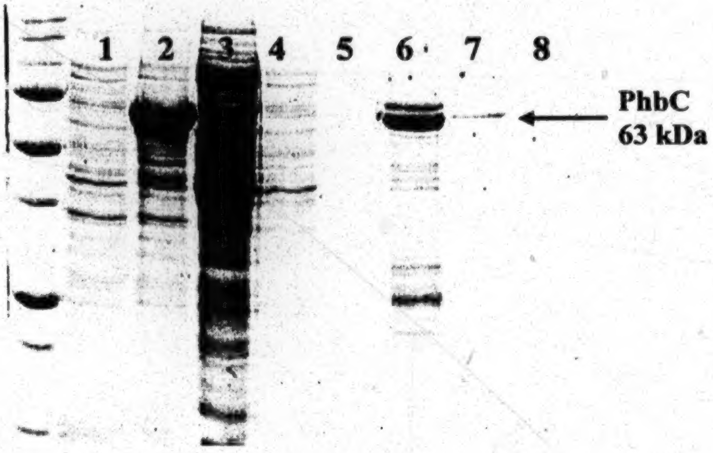


Figure 3-7. Purifications of recombinant PhbA and PhbB proteins

SDS-PAGE gels of purification process of: a.) recombinant PhbA, b.) recombinant PhbB. In both gels, lanes 1 to 8 represent samples collected and prepared from: 1.) cell culture prior to induction (negative control), 2.) cell culture at 4 hours post-induction (positive control), 3.) cell lysate supernatant fraction from T_4 cell culture, 4.) flow-through fraction of cell lysate, 5.) first wash fraction through the column, 6.) second wash fraction through the column, 7.) first elution fraction, 8.) second elution fraction. Arrows indicate target PhbA and PhbB proteins of sizes 44 kDa and 25 kDa.

a. PhbC purification under native condition



b. PhbC purification under denaturing

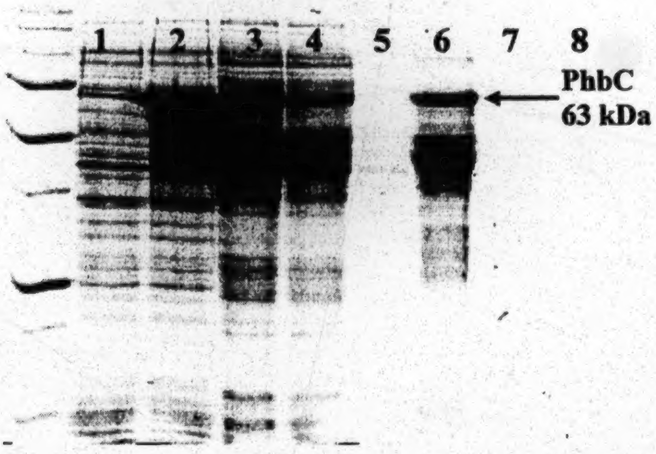


Figure 3-8. Purifications of PhbC protein under native and denaturing conditions SDS-PAGE gels of purification process of recombinant PhbC: a.) under native condition, b.) under denaturing condition. In both gels, lanes 1 to 8 represent samples collected and prepared from: 1.) cell culture prior to induction (negative control), 2.) cell culture at 4 hours post-induction (positive control), 3.) cell lysate supernatant fraction from T_4 cell culture, 4.) first wash fraction through the column, 5.) second wash fraction through the column, 6.) first elution fraction, 7.) second elution fraction and 8.) third elution fraction. Arrows indicate target PhbC protein of size 63 kDa.

As discussed earlier, PhbC is a granule-associated type of protein. It forms inclusion bodies attached to the cell membrane when its concentration becomes higher than its saturation point. This property greatly interfered with the solubility of PhbC. Several efforts were made in order to increase efficiency of cell lysis. More concentrated lysozyme and Triton-X were added to lysis buffer with additional sets of sonication. Attempts to purify PhbC protein were carried out under both native and denaturing conditions. Cell lysate of 4 hours post-induction showed a high expression level of PhbC (lane 2) compared to samples collected prior induction (lane 1) (Figure 3-8a and b). Under a native condition, supernatant from cell lysate (lane 3) did not show a target band with expected intensity. This result suggested a failure in the solubilization procedure, because most of the PhbC protein remains insoluble and still attached to the cell membrane in cellular debris fraction. Lane 6 of the native purification gel (Figure 3-8a) showed a moderate target PhbC band with an expected size slightly above 63 kDa, and several minor bands from the first elution. Fractionation of the second elution in lane 7 showed a faint band of PhbC with no dominant contaminating bands. In lane 8, a fraction of the third elution did not have any protein bands, indicating that the elution was complete.

In order to improve the solubility of PhbC inclusion bodies, cell lysis and purification of PhbC was performed under denaturing conditions following the trouble shooting solution recommended by the kit (QIAexpress Ni-NTA Fast Start). The denaturing lysis buffer was suggested by the manufacturer to have better capability to solubilize inclusion bodies and can completely solubilize His-tag proteins. Therefore under a denaturing condition, His-tag on the target protein would be fully exposed to raisin in the nickel column. This could help improve the efficiency of the purification procedure by increasing the binding of target protein as well as reducing the potential of non-specific bindings. However, the results obtained from denaturing purification were quite contradictory (Figure 3-8b). Fractions of the supernatant obtained from cell lysis (lane 3) showed no difference compared to the lysate of the native purification. Fractions of the flow-through wash buffer (lane 4) showed a moderate size of the target band, which implied a loss of PhbC due to inefficient binding. The first elution fraction (lane 6) showed several minor bands, which are larger than those on the native gel. However there are no contaminated bands appeared in fraction of second washing step (lane 5). This could be resulted from non-specific bindings of contaminated proteins to the column or directly to PhbC itself.

3.3.4 Quantification and immunization of PhbA and PhbB

Concentrations of purified PhbA and PhbB in native elution buffer were investigated following a modified Bradford assay. A standard curve was drawn using average absorbance values at 595 nm of two sets of BSA standards (0, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 µg/µL). The standard curve revealed a linear relation between the concentrations of protein sample (µg/µL) and the absorbance values corresponding to "ABS 595nm = 0.3989 (concentration µg/µL) + 0.0282" equation with an R-squared value of 0.9838. Purified and concentrated PhbA and PhbB, both from the first elutions, were diluted to 1:10 and 1:5 in the same elution buffer, and were prepared by the same method as used for BSA standards (refer to 3.2.8). Two duplicates of each sample were prepared and absorbance values were measured at 595 nm. The concentration of PhbA and PhbB were calculated based on the average absorbance values of 1:10 and 1:5 diluted samples, and the linear relation obtained from BSA standard curve. Purified PhbA samples from the first elution were quantified as 5.023 µg/µL and that for PhbB was 4.336 µg/µL.

In order to produce polyclonal antibodies against PhbA and PhbB for future use in Western blot analysis, purified and quantified protein samples were prepared to meet the immunization criteria in rabbits. Removal of the His-tag and S-tag is normally unnecessary because they are uncharged at physiological pH, rarely alter or contribute to protein function or immunogenicity, and rarely interfere with protein structure (Stiborova et al., 2003). Therefore purified PhbA and PhbB proteins with fusion tags were directly used for downstream immunization process. Since the elution buffer used in purification step was claimed by the manufacturer (personal communication) to be non-harmful to animal hosts, and ready for immunization, protein samples were diluted in the same elution buffer to a final concentration of 1 mg/mL. Two milligrams (2 mg) of PhbA and

PhbB were sent out in dry-ice to University of Calgary SACRI Antibody Services along with SDS-PAGE gel pictures of purified proteins as a proof of quality. PhbA and PhbB were each injected into 2 separate rabbit hosts following a standard 70 day rabbit protocol designed by SACRI antibody services of University of Calgary. The rabbits would be given 5 subsequent injections on day 1, 14, 28, 42 and 56. Blood sample of each rabbit were collected for ELISA test until a maximal level of production obtained.

3.4 Future Work

In this study recombinant PhbA and PhbB proteins were successfully produced and purified using nickel column. Purification of PhbC encountered some difficulties due to insolubility of the protein itself. PhbC forms inclusion bodies at the saturation point, which gets attached to bacterial cell membrane. Several attempts, which included a higher concentration of lysozyme used, sonication to help breakdown of bacterial cells, and purification under a denaturing condition, were undertaken to help improve solubilizing these inclusion bodies. However, satisfactory results were not achieved. Addition of 6 to 8 M urea into cell lysis buffer was proposed to help increase solubilization efficiency. Since PhbC starts to form inclusion bodies after saturation, it is also important to identify induction time at which PhbC starts to get saturated. This can be done by a time-course induction study, in which sample culture is collected every 30 minutes post-induction, and inspected for solubility of the protein. Furthermore media with different components as well as IPTG concentrations added to induce the expression can be adjusted with a combination of the length of inducing time, and the speed of shaking to determine an optimum condition for soluble PhbC production.

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Chapter 4 Production and molecular analysis of transgenic *Arabidopsis*

4.1 Introduction

4.1.1 Mechanism and beneficial uses of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a species of bacteria that causes crown gall disease of a wide range of dicotyledonous plants. This Gram-negative bacterium causes the disease by inserting a small segment of DNA, also known as transfer DNA (T-DNA), into the plant cell, which is incorporated at a semi-random location into the plant genome.

The T-DNA inserted by *Agrobacterium* contains genes for plant growth hormones, which result in tumor production. The T-DNA also contains genes encoding enzymes, which cause the plant to produce specialized amino acids which the bacteria can metabolize, called opines (Zupan et al. 2000). Opines are a class of chemicals that serve as a source of energy for *Agrobacterium*, but not for most other organisms. Most of the genes involved in crown gall disease are not carried on the chromosome of *Agrobacterium* but on a large tumor-inducing plasmid (Ti plasmid) in the bacterial cells. This large Ti plasmid with a size of approximate 200 kb plays fundamental roles in the pathogenesis and the colonization. It harbors not only T-DNA, but also many genes essential for infection and T-DNA transfer to plant cells and genes for catabolism of the opines. *Agrobacterium* strains that contain the Ti plasmid respond strongly to phenolic compounds such as acetosyringone released from wound sites of the plants. Thus, one of the functions of the Ti plasmid is to encode for receptor specific to components that are inserted in the bacterial membrane and enables the bacterium to recognize wound sites. At higher concentrations of 10^{-5} to 10^{-4} molar, acetosyringone triggers the activation of virulence genes (*vir* genes) on the Ti plasmid. These *vir* genes have important roles in the infection process because they control the production of an endonuclease that excises and releases the T-DNA. T-DNA is then transferred into the plant cells, where it integrates into the plant chromosomes and dictates the functioning of those cells (Gelvin 2003).

The DNA transmission capabilities of *Agrobacterium* have been extensively utilized to insert foreign genes into plants. The plasmid T-DNA that is transferred to the plant is an ideal vehicle for genetic engineering (Zambryski 1983). This is done by disarming the T-DNA segment of hormone biosynthesis genes, which causes a plant disease, and replacing it with a desired gene along with marker resistance gene that will be inserted into the host. Because the Ti plasmid is too large to manipulate easily *in vitro*, *vir* genes are inserted into a virulence plasmid that provides virulence function, and T-DNA region in another vector called a binary vector (Figure 4-1). These two modified plasmids are used together in the binary plasmid transformation system (Barton and Chilton 1983). This binary plasmid is a conjugative plasmid that can be transferred from one cell to another. To transfer a binary plasmid vector, which carries the gene of interest from *E. coli DH5α* into *Agrobacterium tumefaciens* strain; triparental mating method is often used (Ditta et al. 1980). Triparental mating allows plasmids to be constructed and propagated in *E. coli* DH5α, and then mated with a helper strain containing a broad-host range plasmid such as pRK2013. The helper plasmid is an important factor, which helps transferring the target binary plasmid vector into *Agrobacterium*. Once the helper plasmid has conjugated into *E. coli* DH5α, it can now mobilize the engineered binary plasmid into *Agrobacterium,* which already contains virulence plasmid essential for T-DNA transmission. The successfully conjugated *Agrobacterium* with both binary and virulence plasmid are screened for presence of selectable makers of both plasmids.

4.1.2 *Arabidopsis* floral dip transformation and molecular characterizations of transgenic plants

Agrobacterium-mediated *Arabidopsis* floral dip transformation was developed in 1994 (Chang et al. 1994, Katavic et al. 1994). Wild type plants are grown until the flowering stage and dipped in *Agrobacterium* suspension carrying Ti-plasmid construct of interest, which includes the target gene and a marker gene. Mature seeds produced from floral dip plants are used in the process of screening for transformed plants. This process uses a selection agent, usually an antibiotic- or an herbicide-resistant marker gene, incorporated initially into the modified gene construct. Non-transformed seedlings, not containing the resistant marker gene, are unable to survive under the selection pressure deliberately built into the experimental design for the selection of transformed target plants. Progeny of parental plants, which have passed the selection process of antibiotic resistance, are subsequently characterized for activity of the introduced transgenes by

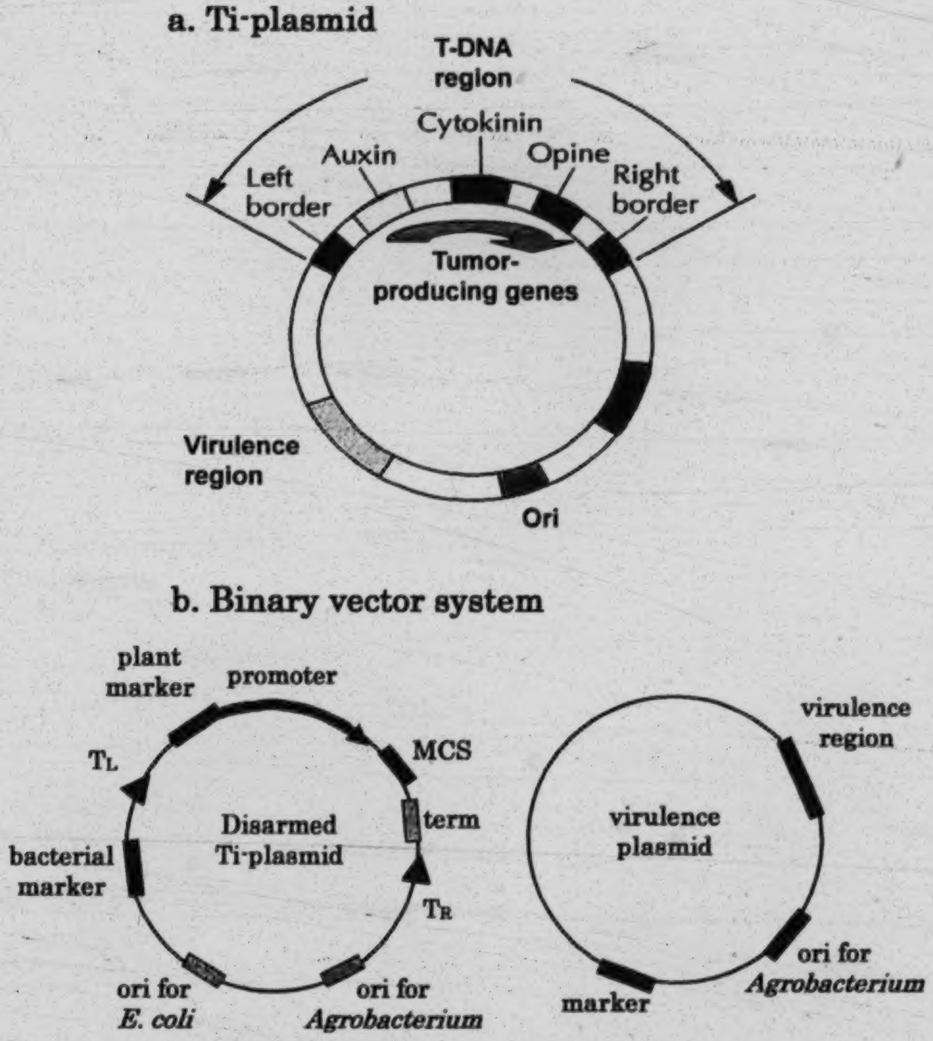
a. Ti-plasmid
T-DNA region
Cytokinin
Auxin
Opine
Left border
Right border
Tumor-producing genes
Virulence region
Ori
b. Binary vector system
plant marker
promoter
MCS
T_L
term
Disarmed Ti-plasmid
bacterial marker
T_R
ori for *E. coli*
ori for *Agrobacterium*
virulence region
virulence plasmid
ori for *Agrobacterium*
marker

Figure 4-1. ***Agrobacterium*** **Ti plasmid and binary vector system**
a.) Ti-plasmid: T-DNA region is divided into three parts, left and right boarders, and the segment of hormone biosynthesis genes. Virulence region carrying *vir* genes and origin of replication (ori) are located outside T-DNA boarders. b.) Binary vector system: disarmed Ti plasmid with removed T-DNA segment that causes a plant disease, and inserted with target gene cassette, and marker resistance gene within left and right boarders (T_L and T_R). Virulence plasmid contains virulence region, origin of replication and marker resistance gene.

several molecular analysis methods (Bent 2000). Initially rapid-PCR is performed using template from genomic DNA of resistant plants to confirm integration of the transgene into plant genome. Numbers of the transgene insertion can be identified from segregation ratio of the marker gene as well as by Southern hybridization using the transgene as a probe. Transcription of the transgene into mRNA can be investigated by reverse transcription-PCR utilizing total RNA extracted from a specific target tissue for cDNA synthesis. The translation of the target gene into protein can be identified by Western hybridization with the anti-target protein antibodies.

4.1.3 Summary

#92phbB:phbC:pKYLX-NAP double-cassette construct, previously prepared for introduction of the PHB synthesis into cytosol of developing seeds, was conjugated into *Agrobacterium* strain GV3101 by triparental mating method using the *E. coli* carrying helper plasmid pRK2013. Presence of the gene construct in the conjugants was confirmed by restriction analysis with appropriate endonuclease digestion of the plasmid DNA isolated from the *Agrobacterium*. Transformation of *Arabidopsis* was performed by floral dip method utilizing the transformed *Agrobacterium*. Mature seeds harvested from parental plants were germinated on 1/2MS plates containing Kanamycin selection in order to screen for seedlings with target gene construct. Presence and copy numbers of the inserted genes (*phbB* and *phbC*) were identified by PCR analysis and segregation ratio of progenies, and southern hybridization. Further molecular analysis of expression of the transgenes was performed by reverse transcription-PCR.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

Agrobacterium tumefaciens strain GV3101 carries a disarmed virulence plasmid pMP90, which is needed for induction of T-DNA. GV3101 was grown on LB plates or liquid media with Rifampicin 50 µg/mL and Gentamycin 20 µg/mL at 28°C. For long term storage, culture grown for over two nights was kept at -80°C with additional glycerol to a final concentration of 10% (v/v).

E. coli HB101 carrying a pRK2013 helper plasmid, is essential to transfer plasmids from the donor *E. coli* strain to the recipient *Agrobacterium* strain. This helper bacteria strain was grown on LB plates or liquid media with Kanamycin selection (100 μg/mL).

4.2.2 Triparental mating method of *Agrobacterium*

Agrobacterium GV3101 was inoculated in 4 mL of LB liquid media (Rifampicin 50 μg/mL, Gentamycin 20 μg/mL) and grown for two days at 28°C. Helper strain carrying pRK2013 plasmid and DH5α strain carrying construct of interest were inoculated in 4 mL of LB liquid media with Kanamycin 100 μg/mL and Tetracycline 15 μg/mL, respectively, and grown at 37°C overnight. Cell pellet of *Agrobacteria* was collected from 1.5 mL culture by centrifugation at 6,000 rpm for 2 min at RT. The media was discarded and 1.5 mL of helper strain culture was added to the *Agrobacteria* pellet. The cell pellet of the helper strain was collected as well by centrifugation. In the same tube, *E. coli* DH5α cells carrying the gene construct of interest was collected from 1.5 mL of overnight grown culture. 1.5 mL of plain LB media was added to the three pellets, mixed by pipetting, and centrifuged at the same speed. The media was discarded to remove antibiotics. The mixed pellet was resuspended in 200 μL of plain LB media, dropped onto the surface of a plain LB plate, and incubated overnight at RT. The mass of bacteria was then scraped and suspended in 200 μL LB. Serial dilutions (10^{-2}, 10^{-3} and 10^{-4}) of the cell suspension were plated on LB plates with Rifampicin 50 μg/mL, Gentamycin 20 μg/mL, and Tetracycline 15 μg/mL and incubated at 28°C for 3-5 days. To ensue that only conjugant *Agrobacterium* cells with the construct of interest were grown on the plates, selected single colonies were re-streaked on a fresh plate with three antibiotics, incubated at 28°C for 2-3 days. This step was repeated for at least 3 times.

4.2.3 *Agrobacterium*-mediated floral dip transformation

Wild type *Arabidopsis* plants (ecotype WS) were grown in a growth chamber as described in 2.2.2 until flowering stage (2-3 weeks). The floral dip transformation was performed following protocol adapted from Clough and Bent, 1998. First bolts of the plants were clipped to encourage proliferation of many secondary bolts. The plants would become ready for transformation generally 6-7 days after the clipping, when they have mostly flower buds and only few silliques. *Agrobacterium* carrying gene construct of interest, obtained from triparental mating, was cultured in 400 mL LB liquid media with appropriate antibiotics at 28°C for 2 days. *Agrobacterium* cells were collected by centrifugation at 4,000 rpm for 15 min at RT. The pellet was resuspended in 400 mL of sterile 5% sucrose (w/v) solution, with an addition of Silwet L-77 to a final concentration of 0.05% (v/v). The above-ground parts of the wild type plants were dipped into the cell suspension for 3 seconds with gentle agitation. Plants were then wrapped with saran wrap in a dome shape to maintain humidity and kept in a growth chamber. The wrap was removed after 2 days, and the plants were grown in the growth chamber, watered as required until seeds became mature. Dry seeds were harvested for further analysis.

4.2.4 Analysis of *Arabidopsis* seedlings

Dry mature seeds were surface sterilized with 70% ethanol (v/v) for 2 min, and with 30% bleach (v/v) for 15 min for 2 times. Then the seeds were rinsed thoroughly twice with sterile distilled water. These seeds were then resuspended in sterile 0.1% agarose (w/v) and plated onto 1/2MS plate (1/2 MS, 1% sucrose, 0.8% tissue culture grade phytagar, pH 5.6) with Kanamycin (100 µg/mL). The plates were cold-treated at 4°C for 2 days for uniformity of seed germination, and transferred to tissue culture room that runs at 25°C 18 hours 100 mE photoperiod. 10 day-old seedlings were observed for successful transformants. Transgenic seedlings that have resistance to Kanamycin in the media were healthy and green, while those with no resistance appeared smaller and pale. Transformed seedlings (T_0 plants) were transplanted into soil. The roots of the seedlings were gently washed with distilled water to remove the media before transplanting into soil. The plants were covered with saran wrap to keep moisture inside, and to let them recover from the transplant shock. They were kept in the growth chamber and watered as needed. The saran wrap was gradually opened to let the plants acclimatize to condition of the growth chamber, where they were kept until mature seeds (T_1) were ready for harvesting.

4.2.5 Rapid genomic DNA extraction and PCR analysis

Genomic DNA extraction of *Arabidopsis* was performed following the protocol described by Kasajima et al. (2004). A piece of rosette leaf (3-5mg) was placed in a 1.5 mL tube with 20 μL of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) and 180 μL of TE buffer (10mM Tris-HCl pH 8.0, and 1mM EDTA). The leaf was crushed with a sterile plastic rod against the tube walls until fine tissue residues were obtained. 1 μL of DNA extraction was added to 20 μL of PCR mixture. PCR reactions and gel-electrophoresis were performed as described in 2.2.5.

4.2.6 Genomic DNA extraction for southern blot analysis

Genomic DNA from *Arabidopsis* leaf was extracted using Promega Wizard Genomic DNA Purification Kit (Promega Co., Nepean, ON). 60 mg of frozen *Arabidopsis* leaf tissue was ground to fine powder in liquid nitrogen with sterile mortar and pestle. The powder was transferred into 1.5 mL tube with 600 μL of nuclei lysis buffer. After vigorous vortex, the suspension was incubated at 65°C for 15 min. 3 μL of RNase solution was added, followed by incubation at 37°C for 15 min. After cooling down to RT, 200 μL of protein precipitation solution was added to the suspension and vortexed for 20 sec. Proteins and other impurities were removed by centrifugation at 16,000 x g for 3 min at RT. Supernatant was transferred into a fresh tube, containing 600 μL isopropanol. The solution was mixed by inverting the tube several times, and centrifuged again at the same speed for 1 min. After discarding the supernatant, precipitated DNA pellet in the tube was washed twice with 600 μL of 70% ethanol (v/v). The pellet was air-dried at RT, dissolved in 50 μL DNA rehydration buffer, and incubated at 65°C for 1 hour. 1 μL of this extracted and purified genomic DNA solution was electrophoresed on 0.8% agarose gel to confirm the quality, quantity and molecular weight of the sample.

4.2.7 Southern Hybridization

20-30 μg of genomic DNA was digested with 40 units of restriction endonuclease overnight at 37°C. On the next day, other 40 units of endonuclease were added for further digestion for 6-7 hours. The digestion reaction was stopped by inactivation of the endonuclease at 65°C for 15 min. Total volume of reaction solution was electrophoresed on 0.8% agarose gel in TBE buffer (89 mM Tris-HCl, 89 mM Boric acid, 20 mM EDTA, pH 8.0) at 45 voltage overnight or until the marker band had migrated two thirds of the gel length. Excess parts of the gel and the top right corner were trimmed off as a mark of orientation, and the size of the gel was measured. The gel was depurinated in 11.6 M HCl for 10 min at RT with gentle shaking, rinsed with distilled water, followed by denaturation in 1.5 M NaCl and 0.5 N NaOH for 45 min at RT to separate double-stranded DNAs into single strands. The gel was then rinsed in distilled water, neutralized in 0.5 M Tris-HCl, pH 7.5 and 1.5 M NaCl for 30 min at RT, and rinsed again with distilled water. Single-stranded DNA fragments were transferred overnight from the gel onto Hybond N+ nylon membrane (Amersham Inc., Baie d'Urfe, QC) by a sandwich blot between two pieces of filter paper (Whatman Inc., Florham Park, NJ) soaked in 1xSSC transfer buffer (150 mM NaCl, 15 mM Sodium Citrate, pH 7.2) as described by Sambrook et al. (1989). DNA transfer was also done with 1xSSC buffer. The DNA fragments were fixed on the blot by U.V. crosslink. Pre-hybridization was performed by incubating the blot in BEPS pre-hybridization buffer (1% BSA (w/v), 1 mM EDTA, 0.5 M Na_2HPO_4, 7% SDS (w/v), pH 7.2) at 65°C for at least 2 hours. Labeled probe was prepared separately using radioactive (^{32}P) and non-radioactive (fluorescein) materials by Random Prime DNA Labeling Kit (Invitrogen) and AlkPhos Direct Labeling and Detection System with ECF^{TM} substrate (Amersham) respectively. Denatured DNA probe was added into pre-hybridization solution containing the membrane, and hybridization was continued overnight at 65°C. The blot with ^{32}P labeling was then washed once with 0.1% SDS in 5xSSC buffer (750 mM NaCl, 75 mM Sodium Citrate, 0.1% SDS (w/v), pH

7.2) and twice with 0.1% SDS in 2xSSC buffer (300 mM NaCl, 30 mM Sodium Citrate, 0.1% SDS (w/v), pH 7.2) at 65°C for 30 min each. The blot was wrapped with saran wrap, and was exposed to an X-ray film to detect signals. The blot with fluorescein labeling was washed twice with a primary wash buffer (2 M Urea, 0.1% SDS (w/v), 2% blocking reagent (w/v), 0.5 M NaH_2PO_4, 1 mM $MgCl_2$) at 55°C for 20 min and twice with a secondary wash buffer (50 mM Tris-HCl, 100 mM NaCl, pH 10) at RT for 10 min each. The signal detection was performed by adding ECF substrate, for alkaline phosphatase enzyme conjugated to anti-fluorescein antibodies. The blot was kept overnight in a dark place at RT, and signals were detected using the Typhoon TR10+ Variable Mode Image scanner equipped with Image QuantTL software (Amersham).

4.2.8 Total RNA extraction

Total RNA was extracted from developing seeds using RNeasy Mini Kit (Qiagen). Seed pods with developing seeds inside were collected and immediately put into liquid nitrogen. 80 mg of frozen tissue (seeds in fruits) was ground with sterilized and baked (at 250°C for 10-12 hours) mortar and pestle in liquid nitrogen to fine powder. The frozen powder was transferred into a 2 mL tube with 450 μL lysis buffer containing guanidine thiocyanate (GITC) and β-Mercaptoethanol which rapidly inactivates endogenous RNase, and n-lauroyl sarcosine to denature ribonucleoprotein complexes. The lysate was passed through a QIAshredder spin column and centrifuged to remove cell debris and homogenized to shear genomic DNA. The supernatant of the flow-through fraction was transferred into a fresh tube, mixed with pure ethanol, and passed through an RNeasy spin column, which has a silica-gel membrane that adsorbs total RNA, by centrifugation. Total RNA bound onto the silica-gel membrane was treated with DNase (RNase –Free DNase Set, Qiagen) by adding 80 μL of DNase solution directly onto the membrane in the spin column and incubated at RT for 20 min. Digested DNA and other contaminants were washed off three times with wash buffer by centrifugation. Total RNA was eluted from the silica-gel membrane with 10 μL RNase-free water. 2 μL of each sample was electrophoresed on 1% agarose gel to confirm the quality, quantity and molecular weight of extracted RNA.

4.2.9 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR reaction was performed using solutions and a protocol supplied by OneStep RT-PCR Kit (Qiagen). RNA template and primers used were prepared in RNase-free water. RT-PCR reaction was performed with a mixture of following components: 5 μL 5xOneStep buffer, 1 μL dNTP mix (10 mM each), 2 μL forward primer (10 μM), 2 μL reverse primer (10 μM), 2 μL RNA template, 5 μL 5xQ solution, 0.1 μL RNase inhibitor (40 units/μL, Qiagen), 1 μL enzyme mix (Omniscript reverse transcriptase with Sensiscript reverse transcriptase and HotStarTaq DNA polymerase, Qiagen), and volume up to 25 μL with RNase-free water. Reverse transcription for cDNA synthesis was performed at 50°C for 30 min. Amplification steps were performed with a hot start at 96°C for 15 min, and then 40 cycles of [96°C for 40 sec, 52°C-54°C for 40 sec, and 72°C for 1-2 min depending on primers, templates and size of expected products], followed by an extension step at 72°C for 10 min. The amplified RT-PCR products were subsequently electrophoresed on a 0.8%-1% (w/v) agarose gel at 80-100 voltage according to the size of the PCR fragments.

4.2.10 Analysis of fatty acid profile of seed lipids

Fatty acid composition of total acyl lipid from mature seeds was determined following the International Organization for Standardization method reference number ISO 5508:1990 (E), "Animal and vegetable fats and oils—Analysis by GC of methyl esters of fatty acids". 50 mg of mature *Arabidopsis* seeds were crushed in 1 mL petroleum ether in a 5 mL polypropylene vial using a steel rod. After allowing the meal to settle, 0.5 mL of supernatant was transferred to a glass tube containing 1.2 mL methylating solution (2% sodium methoxide in methanol (w/v)). After thorough mixing, the solution was incubated at RT for 30 min. 1mL sterile distilled water was added to the solution, mixed well and left for 10 min at RT for the phases to separate. After separation, 200 μL from the upper layer was diluted with 300 μL petroleum ether in a GC autosampler vial and 1 μL was injected into a GC column.

Separation of fatty acid methyl esters (FAMEs) was performed on a flame ionization gas chromatograph (model 6890, Hewlett Packard, Mississauga, ON) fitted with a 30-m X 0.25 mm (i.d.) column (HP-INNOWAX, crosslinked polyethylene glycol) with helium as the carrier gas at a flow rate of 28.0 mL/minute. The oven temperature was from 180°C to 230°C at a rate of 5°C/minute and hold at 230°C for 13 minutes. Peaks were assigned by comparing retention time of those of FAME standards and relative proportions of FAMEs were determined as percentages of summed peak areas.

4.3 Results and Discussion

4.3.1 Triparental mating *Agrobacterium* transformation

Agrobacterium strain GV3101 was transformed with #92phbB:phbC:pKYLX-NAP cytosolic double-cassette construct, which carries *phbB* and *phbC* genes isolated from *R. eutropha*, by a triparental mating method described in 4.1.1. Three single *Agrobacterium* colonies were randomly selected after triparental mating, named A to C, and individually streaked on LB plates with antibiotic selections. The plates were incubated at 28°C for 2-3 days. A single colony from each master plate were re-streaked and named 1A, 1B, and 1C. This step was repeated 4 times until obtained single colonies 4A, 4B, and 4C, which were derivatives of the master A, B, and C colonies. The re-streaking process was performed to ensure that only the transformed *Agrobacterium*, which contains the plasmid of interest, survives. If the re-streaking was only done once, some of the *E. coli* cells that attached to the *Agrobacterium* colony may have a chance to survive via the nutrients and resistances to antibiotics provided by the *Agrobacterium* colony.

Colonies 4A, 4B and 4C were cultured in LB liquid media with Rifampicin, Gentamycin and Tetracycline selections at 28°C for 2 nights. Plasmid DNA was isolated from 20 mL of culture from each clone using Miniprep Extraction Kit (Qiagen) following low-copy plasmid purification protocol. Restriction analysis with HindIII and XbaI digestion was performed in order to confirm presences of the transgenes (*phbB* and *phbC*). Digested products of clone 4A plasmid DNA showed bands with expected sizes

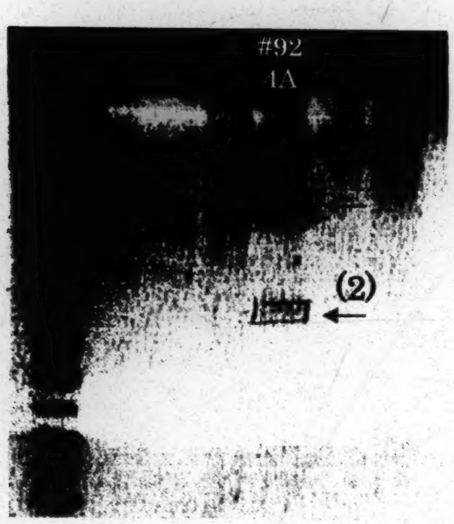

Figure 4-2. Restriction analysis of insert genes on #92phbB:phbC:pKYLX-NAP of *Agrobacterium* clone 4A
Plasmid DNA was extracted from *Agrobacterium* clone 4A after triparental mating, and digested with HindIII and XbaI. Arrows (1) and (2) indicate *phbC* and *phbB* bands with sizes of 1.8 kb and 0.7 kb, respectively.

of 0.7 kb and 1.8 kb corresponding to sizes of *phbB* and *phbC* respectively (Figure 4-2). This clone was then re-cultured and stored at -80°C.

4.3.2 *Arabidopsis* transformation with the cytosolic expression construct

Wild type *Arabidopsis* plants were grown in a growth chamber as described in 2.2.2. Total 6 pots of *Arabidopsis* with each containing 50 to 100 plants at the flowering stage were used in the transformation. Clone 4A of *Agrobacterium* strain carrying #92phbB:phbC:pKYLX-NAP binary vector for introducing PHB synthesis into cytosolic compartments in developing seeds was cultured in 400 mL LB media with Rifampicin (50 μg/mL), Gentamycin (20 μg/mL) and Tetracycline (15 μg/mL) selections for 2 days. *Agrobacterium* cells collected by centrifugation and resuspended in 400 mL sterile 5% sucrose (w/v) with 0.05% Silwet L-77 (v/v) solution were used to transform *Arabidopsis* plants as described in 4.2.3. Transformants were selected on Kanamycin plates as described in 4.2.4. Twenty-two green healthy T_0 seedlings were named 92-1 to 92-22, and carefully transferred into soil as described in 4.2.4. Transformed plants were maintained in the growth chamber for a few weeks until mature seeds (T_1) were ready for harvesting.

4.3.3 Transgene copy number determination

T_1 seeds harvested from 22 Kanamycin resistant plants were analyzed for copy numbers of transgenes. Since the two transgenes, *phbB* and *phbC*, were inserted within left and right boarders of the same Ti plasmid, a successful transformation with this double-cassette construct should result in equal copy numbers for both transgenes in the transformant. According to Mendel's law of segregation, progenies derived from self-pollinated heterozygous parents of one locus (Aa) exhibit dominant to recessive phenotypes of a 3 :1 ratio (AA, 2Aa, aa). In this case, *nptII* is considered as a dominant allele (A), which gives a dominant phenotype of resistance to Kanamycin. Thus T_1 progenies derived from self-pollinated heterozygous T_0 parents (Aa) with a single copy of transgenes (A) would have Kanamycin resistant and susceptible types of a 3:1 ratio. On the other hand, progenies derived from self-pollinated heterozygous transformants with multiple copies of transgenes would not follow 3:1 ratio. For instance, T_0 transformants with transgenes inserted into 2 positions, which in this case considered as different loci (AaBb), would give progenies with phenotypes: 9A_B_, 3A_bb, 3aaB_, and 1aabb. However, since the two dominant alleles A and B would exhibit the same Kanamycin resistant phenotype; the possibility of having one susceptible progeny (aabb) from AaBb self-pollination is 1 in 16, or the ratio of resistant to susceptible progenies is 15:1. Correspondingly progenies from parental plants with higher copy numbers of transgenes would have a higher ratio of individuals with dominant Kanamycin resistant phenotype (e.g. 1 in 64 susceptible for 3 copies).

50-100 mature T_1 seeds collected from each T_0 resistant plants (92-1 to 99-22) were germinated on 1/2MS plates with Kanamycin selection to determine the segregation ratio of the transgenes. 10-day old seedlings from each line were observed and counted for green (resistant) and pale (susceptible) plants. The green to pale ratio of each line as well as the growth condition of the seedlings are given in Table 4-1.

T_1 seedlings from lines 1, 2, 4, 5, 6, 7, 8, 11, 12, 13, 14, 15, 17, 18, 19, 20 and 22 showed the segregation ratios close to 3 (green): 1 (pale), and were predicted to have a single copy of insert genes. Other lines with segregation ratio closer to 15 (green): 1 (pale) or more were considered multiple copy insertion. Eight resistant seedlings from each healthy line with a single copy insertion were transferred into soil, and maintained in the growth chamber for further analysis.

4.3.4 Rapid confirmation of transgenes integration by PCR analysis

In continuation of analysis of transgenic lines for resistance to Kanamycin, it is also important to confirm that not only *nptII* marker gene, but the two target transgenes, *phbB* and *phbC*, have been integrated into the nuclear genome as well. In order to confirm the presence of both transgenes, PCR analysis using genomic DNA as a template was performed. T_1 plants selected from Kanamycin resistant seedlings with single copy transgene insert were transferred into soil and maintained in the growth chamber. Genomic DNA extraction from leaf was performed following a protocol described by Kasajima et al. (2004). PCR analysis of the *phbB* gene was performed using

Table 4-1. Segregation ratios of T_1 seedlings

T_0 parental Lines	Green	Pale	Green : Pale	Predicted copy number	Growth condition
92-1 *	111	23	4.8 :1	1	Contaminated with fungus
92-2 *	79	22	3.5 :1	1	Seedlings are small
92-3	98	6	16.3 :1	2	Healthy
92-4 *	77	20	3.8 :1	1	Healthy **
92-5 *	69	20	3.4 :1	1	Healthy **
92-6 *	105	20	5.3 :1	1	Healthy **
92-7 *	78	19	4:1	1	Seedlings are small
92-8 *	104	29	3.5 :1	1	Seedlings are small
92-9	74	1	74:1	>2	Healthy
92-10	72	5	14.4 :1	2	Healthy
92-11 *	101	20	5:1	1	Seedlings are small
92-12 *	87	22	3.9 :1	1	Seedlings are small
92-13 *	75	16	4.6 :1	1	Healthy **
92-14 *	89	16	5.5 :1	1	Healthy **
92-15 *	62	26	2.3 :1	1	Healthy **
92-16	87	2	43.5 :1	>2	Healthy
92-17 *	78	21	3.7 :1	1	Contaminated with fungus
92-18 *	88	28	3.1 :1	1	Healthy **
92-19 *	58	17	3.4 :1	1	Seedlings are small
92-20 *	57	13	4.3 :1	1	Contaminated with fungus
92-21	79	1	79:1	>2	Healthy
92-22 *	55	14	3.9 :1	1	Seedlings are small

* = lines expected to have a single copy of transgenes

** = healthy lines with a single copy of transgenes transferred into soil for further use

"#19 phbB forward" and "#24 phbBI" reverse primers to amplify 350 bp fragment at 5' end of *phbB*. PCR analysis of *phbC* gene was performed using "#7 phbCF1" forward and "#26 phbCI" reverse primers to amplify 300 bp fragment at 5' end of *phbC*. Positive controls of PCR reactions were performed using #1phbB:pBSK(-) and #2phbc:pBSK(-) plasmid DNA (refer to 2.3.1) as a template. Genomic DNA extracted from wild type plants was used as a template for negative control.

Genomic DNA extracted from 8 resistant plants from each of the lines 92-4, 5, 6, 13, 14, 15 and 18 with a total of 56 samples, were used for PCR analyses of *phbB* and *phbC*. Results of amplified products of *phbB* and *phbC* fragments from the total of 112 reactions of 56 plant samples showed presence of transgenes in every individual (Figure 4-3). The sizes of PCR bands were corresponding to sizes of positive control bands. PCR using genomic DNA template from wild type plant did not show any amplified products. These results confirmed that *phbB* and *phbC* genes were successfully integrated into transgenic plant genome through floral dip transformation. To reduce the number of plants to maintain and analyze, lines 92-5, 6, 13, and 14 were selected, because of their good growth condition and seed productivity, to continue for further experiments. Each T_1 plant was named by a number followed by the number of parental line. For instance, 8 T_1 plants derived from 92-4 T_0 parent were named 92-4.1 to 92-4.8. Total of 32 T_1 plants were maintained in the growth chamber until T_2 seeds were produced and ready for harvesting.

4.3.5 Identification of homozygous lines

Mature T_2 seeds were harvested from 8 T_1 plants of each of the 4 lines. To identify homozygous T_1 plants, 50-100 T_2 seeds derived from each Kanamycin resistant T_1 plant were germinated on 1/2MS media plates with Kanamycin selection. Progenies derived from self-pollination of homozygous T_1 parents (AA) should all have resistance to Kanamycin. On the contrary progenies of heterozygous T_1 parents (Aa) should show segregation ratio of 3(resistant): 1(susceptible). T_2 seedlings from each T_1 parental plant were counted for green and pale ratio. The results are shown in Table 4-2.

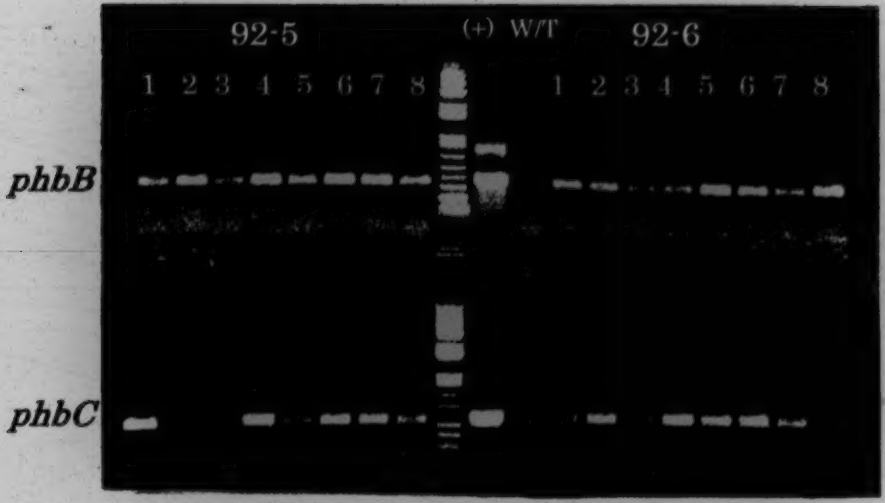

Figure 4-3. Confirmation of transgenes integration of plants from lines 92-5 and 92-6 by PCR analysis
PCR analysis was performed using internal primers to amplify 350 bp fragment of *phbB* and 300 bp fragment of *phbC.* Genomic DNA was extracted from 8 plants of each line, and used as a template for the reaction. The number in each lane indicates the number of each plant. #1phbB:pBSK(-) and #2phbc:pBSK(-) plasmids were used as positive controls (+). Genomic DNA extracted from wild type plant was used as a negative control (W/T).

Table 4-2. Segregation ratios of T_2 seedlings

T_1 parental lines	Green	Pale	Ratio	T_1 parental lines	Green	Pale	Ratio
Line 92-5				Line 92-13			
92-5.1 *	35	0		92-13.1	35	12	2.9 :1
92-5.2	91	20	4.5 :1	92-13.2 *	55	0	
92-5.3 *	106	0		92-13.3 **	10	0	
92-5.4	77	17	4.5 :1	92-13.4	51	30	1.7 :1
92-5.5	cont	a few		92-13.5 *	55	0	
92-5.6	57	15	3.8 :1	92-13.6 **	7	0	
92-5.7	126	33	3.8 :1	92-13.7 *	45	0	
92-5.8 *	110	0		92-13.8 *	34	0	
Line 92-6				Line 92-14			
92-6.1	67	16	4 : 1	92-14.1 *	78	0	
92-6.2	63	22	2.8 :1	92-14.2	79	27	2.9 :1
92-6.3	77	18	4.2 :1	92-14.3	86	28	3 :1
92-6.4	cont	a few		92-14.4	51	18	2.8:1
92-6.5	70	13	5.3 :1	92-14.5 *	64	0	
92-6.6 *	104	0		92-14.6 *	38	0	
92-6.7	44	15	2.9 :1	92-14.7	60	16	3.75 :1
92-6.8	72	18	4 :1	92-14.8 *	90	0	

* = homozygous T_1 parental plant

** = lines with very poor seed germination that there were not enough seedlings to conclude heterozygous/ homozygous.

Cont = seedling plates contaminated with fungus

Segregation ratios of T_2 seedlings showed presences of both heterozygous (Aa) and homozygous (AA) plants in each line. Three of the 8 resistant T_1 plants from line 92-5 (92-5.1, 92-5.3 and 92-5.8) exhibited homozygousity with no T_2 susceptible seedlings. One plant from line 92.6 (92-6.6), 4 plants each from line 92-13 (92-13.2, 92-13.5, 92-13.7 and 92-13.8) and line 92-14 (92-14.1, 92-14.5, 92-14.6 and 92-14.8) were homozygous. The segregation ratios of T_2 seedlings derived from heterozygous T_1 plants were close to 3(green):1(pale), which once again confirmed a single copy transgenes in these lines. Due to poor germinations of T_2 seeds from 92-13.3 and 92-13.6 plants, although there were no susceptible seedlings, total numbers of seedlings were not sufficient to conclude them as homozygous. T_2 seedlings from homozygous lines were transferred into soil and maintained in a growth chamber for tissue harvest and seed multiplication for molecular analysis experiments.

4.3.6 Copy number identification by southern hybridization

Young leaves were collected and pooled from wild type plants and from T_2 homozygous plants of the 4 lines (92-5, 92-6, 92-13, and 92-14), and frozen in liquid nitrogen. 60 mg of the frozen tissue from each line and wild type control were used for genomic DNA isolation, as described in 4.2.6. 1 μL of each DNA sample was electrophoresed on 0.8% agarose gel (Figure 4-4). Genomic DNA from all the plant materials showed high molecular weight with concentration of 500 μg/mL as estimated from intensity of the bands relatively to marker bands. Approximately 20-30 μg of genomic DNA from each line, and wild type negative control, and 2 μg of #7CTP+B:pBSK(-) plasmid positive control were digested with HindIII restriction endonuclease. The digested products were electrophoresed on 0.8% agarose gel, and transferred to nylon membrane as described in 4.2.7. Digested products of genomic DNA from wild type and transgenic plants showed smear bands, which indicated a good digestion, although not possible to conclude as complete digestion (Figure 4-5). The membrane was hybridized with 0.7 kb labeled *phbB* fragment as a probe. After washing, the positive control lane was cut off from the membrane.

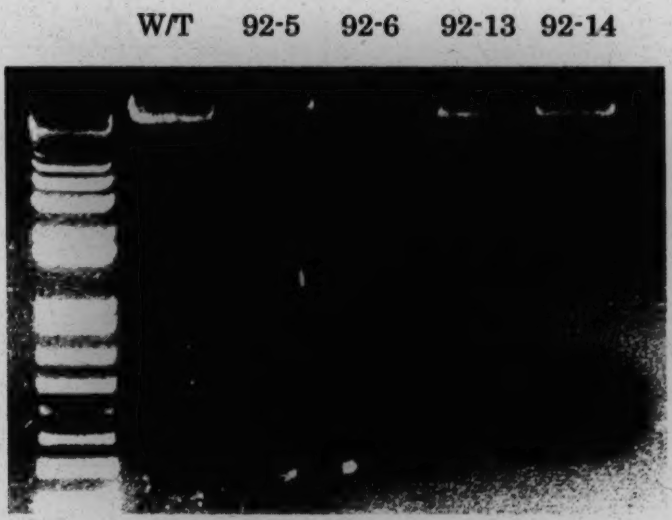

Figure 4-4. Genomic DNA extraction for southern hybridization
Genomic DNA was extracted from leaf tissue of wild type (W/T) and homozygous plants of lines 92-5, 92-6, 92-13, 92-14. The band in each lane shows 1 μL of each DNA sample on 0.8% agarose gel.

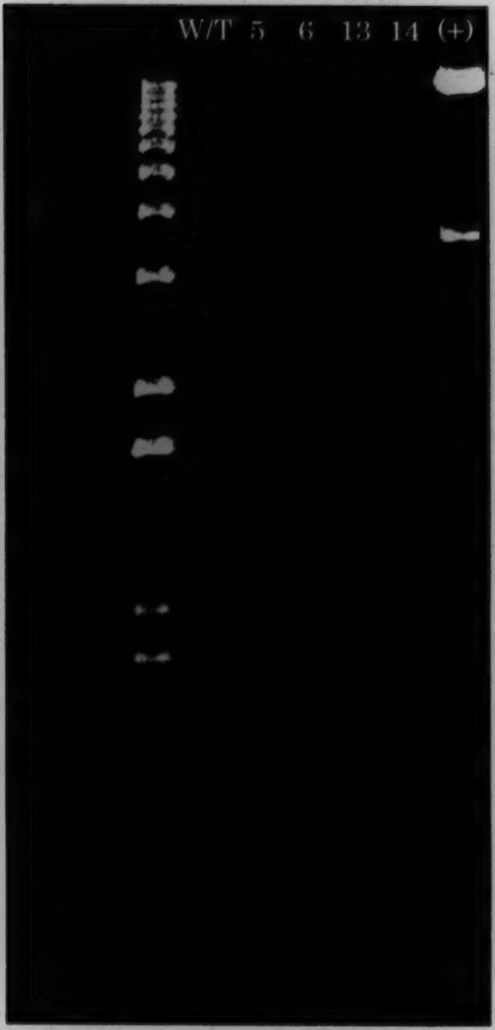

Figure 4-5. Restriction digestion of genomic DNA by HindIII
Digested genomic DNA from wild type W/T and homozygous plants of lines 95-5, 92-6, 92-13 and 92-14 (lanes 5, 6, 13, and 14) were electrophoresed on 0.8% agarose gel., #7CTP+B:pBSK(-) plasmid was used as a positive control (+).

Figure 4-6. Southern hybridization with radioactive probe (^{32}P)
X-ray film of southern hybridization of genomic DNA of wild type (W/T) and homozygous plants from lines 92-5, 92-6, 92-13 and 92-14 (lanes 5, 6, 13 and 14 on the film). #7CTP+B:pBSK(-) plasmid was used as a positive control (+).

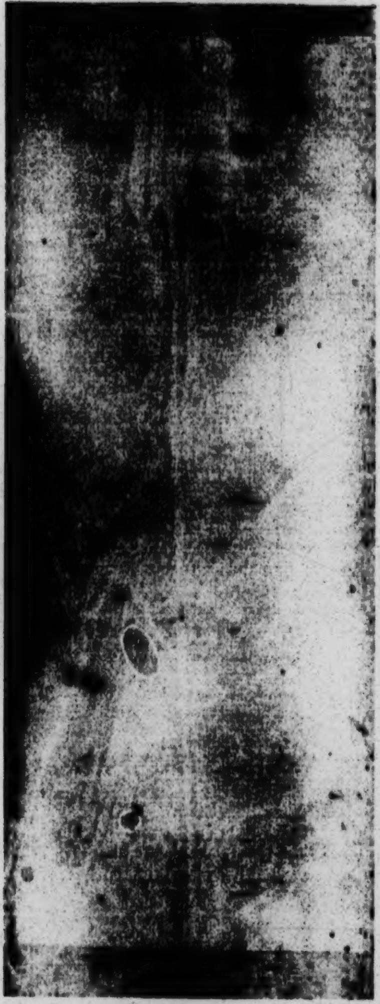

Figure 4-7. Southern hybridization with non-radioactive probe (ECF)
Southern hybridization membrane of genomic DNA of wild type (W/T) and homozygous plants from lines 92-5, 92-6, 92-13 and 92-14 (lanes 5, 6, 13 and 14 on the membrane).

Two pieces of the blot, one with transgenic samples and the other with a positive control, were exposed on the X-ray film for signal detection.

Since the transgenic lines used in this experiment were earlier estimated to carry a single copy of transgene by the segregation ratios, the southern hybridization was expected to give one signal band on each transgenic lane. However, the results did not conform to this expectation (Figure 4-6). As expected, DNA from a wild type negative control in lane WT did not produce any signal. DNA samples from the 4 transgenic lines in lanes 5 to 14 generated several bands on the film. These several signal bands might be the outcome of incomplete digestion of genomic DNA.

Southern hybridization experiment was repeated again twice with reduced amount of genomic DNA to 10 μg to avoid the problem of incomplete digestion, and the label-probe was prepared using a non-radioactive (fluorescein) material by AlkPhos Direct Labeling and Detection System with ECF (Amersham). However the signals were not strong and secondary bands were still present in some lanes (Figure 4-7).

4.3.7 Transgene expression analysis by RT-PCR

Immature green fruits containing developing seeds were collected from T_3 Homozygous plants. Total RNA was extracted from 80 mg of these fruits, which were frozen in liquid nitrogen, using RNeasy Mini Kit (Qiagen). The RNA was treated with DNase solution (RNase-Free DNase Set, Qiagen) to eliminate contaminating DNA. Extracted and purified RNA samples from T_3 homozygous plants of 4 transgenic lines (92-5, 92-6, 92-13, and 92-14) and wild type plant were eluted in 10 μL RNase-free water. To confirm the quality of RNA samples, 2 μL of each sample was electrophoresed on 1% agarose gel (Figure 4-8).

All the RNA samples showed 2 sharp bands. The upper 28S rRNA bands of all samples were approximately twice as intense as the lower 18S rRNA bands by visual estimation. This result indicated that the RNA samples were intact with no degradation. The total RNA samples were used as templates for RT-PCR to detect transcriptions of the target genes to mRNAs. RT-PCR reaction of *phbB* transgene was performed using forward primer "#19phbB Forward", and reverse primer "#6phbB R1" to amplify

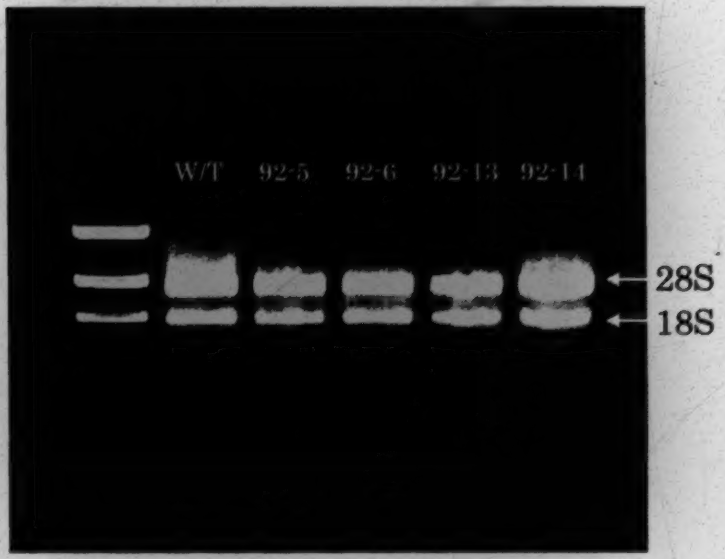

Figure 4-8. Total RNA extracted from young fruits for RT-PCR
Total RNA was extracted from fruits of wild type (W/T) and homozygous plants of lines 92-5, 92-6, 92-13, and 92-14. The band in each lane shows 2 μL of each RNA sample on 1% agarose gel. Arrows indicate upper and lower bands of 28S and 18S rRNA, respectively.

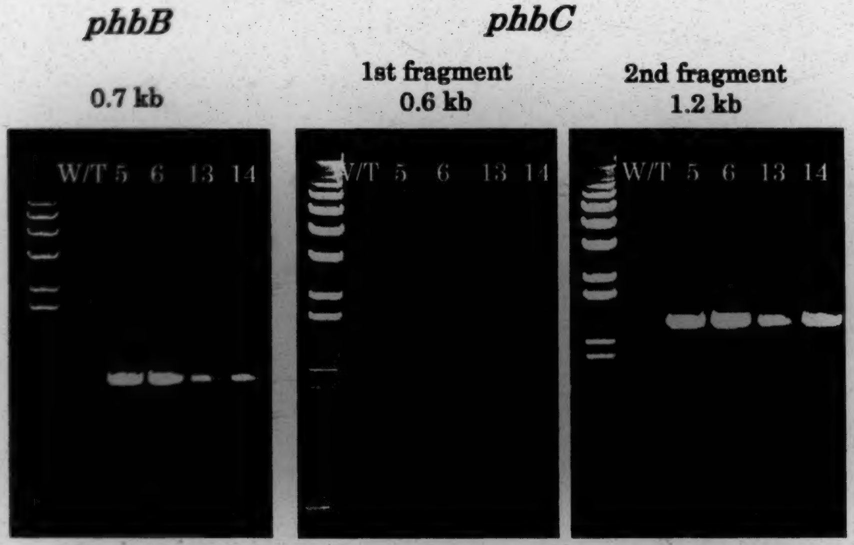
phbB
phbC
0.7 kb
1st fragment
0.6 kb
2nd fragment
1.2 kb
W/T 5 6 13 14
W/T 5 6 13 14
W/T 5 6 13 14

Figure 4-9. Reverse transcription-PCR of *phbB* and *phbC*
Total RNA of young fruits of wild type (W/T) and homozygous plants from lines 92-5, 92-6, 92-13 and 92-14 (lanes 5, 6, 13 and 14) were used in *phbB* and *phbC* expression analysis. RT-PCR using *phbB* primers showed amplified full-length 0.7 kb fragment. RT-PCR of *phbC* was divided in two reactions of the first 0.6 kb fragment and the second 1.2 kb fragment.

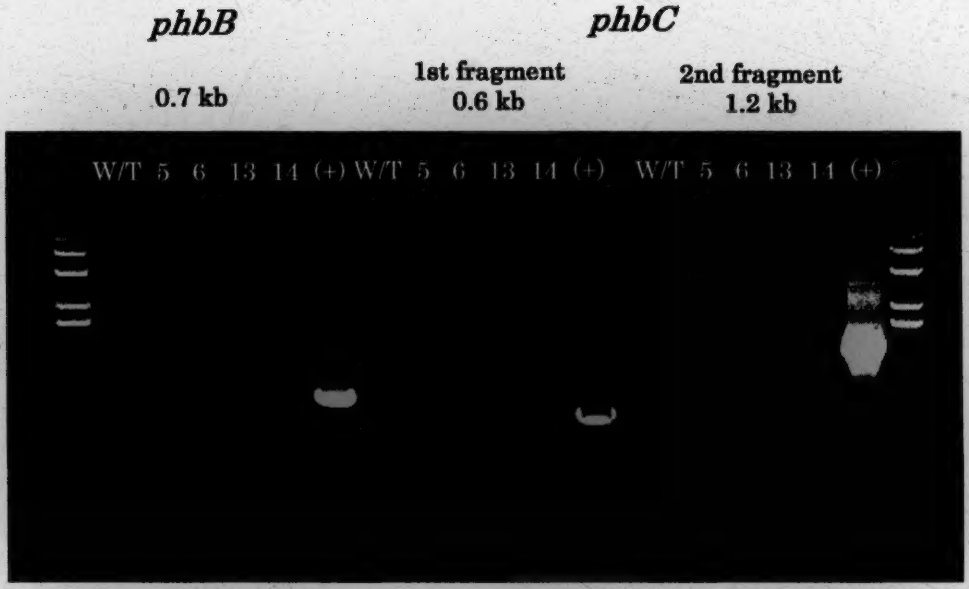
phbB
phbC
0.7 kb
1st fragment
0.6 kb
2nd fragment
1.2 kb
W/T 5 6 13 14 (+) W/T 5 6 13 14 (+) W/T 5 6 13 14 (+)

Figure 4-10. Normal PCR of *phbB* and *phbC* using total RNA templates
Total RNA of young fruits of wild type (W/T) and homozygous plants from lines 92-5, 92-6, 92-13 and 92-14 (lanes 5, 6, 13 and 14) were used as a template for normal *phbB* and *phbC* amplifications. #1phbB:pBSK(-) and #2phbc:pBSK(-) plasmids were used as a positive control (+) for the reactions.

the full-length 0.7 kb fragment from cDNA. RT-PCR reactions of *phbC* were divided in two fragments due to the difficulty encountered in full-length 1.8 kb amplification. The first RT-PCR was performed using forward primer "#7phbCfra1F1" and reverse primer "#8phbCfra1 R1" to amplify the first 0.6 kb of *phbC* from cDNA. The second RT-PCR was performed using forward primer "#9phbCfra2 F1" and reverse primer "#18phbC Reverse" to amplify second 1.2 kb fragment (Figure 4-9). Reverse transcription for cDNA synthesis was performed at 50°C for 30 minutes, followed by amplification of transgenes. Amplified RT-PCR products using RNA samples from transgenic lines showed appropriate bands with expected sizes, while RT-PCR reaction of wild type RNA sample did not give a positive band. These results indicated that *phbB* and *phbC* genes were successfully transcribed into mRNAs in the developing fruits (possibly specifically in seeds) of the transgenic plants. To ensure that the amplified PCR products were derived from cDNA templates, specifically synthesized from mRNA, and not from any contaminated DNA in the total RNA samples, normal PCR reactions were performed using the same primers and RNA templates with omission of cDNA synthesis reaction (Figure 4-10). Amplified products of the positive control using plasmid DNA showed bands with appropriate size to each reaction, while there was no positive product band from any reaction using total RNA templates. These results strongly confirmed that there was no DNA contaminations in RNA extraction as well as the transgenes were properly transcribed in the developing seeds of the transgenic lines.

4.3.8 Fatty acid profile of transgenic seed lipid

Acetyl-CoA, the substrate for PHB synthesis, is also a key compound in other metabolic pathways including fatty acid synthesis. Thus it is also interesting to investigate fatty acid composition of lipids from transgenic seeds in comparison to the composition of lipid from wild type control. 50 mg of mature transgenic seeds harvested and pooled from homozygous plants from each line were used for fatty acid profile analysis by gas chromatography. The seeds were crushed in 1mL petroleum ether to dissolve the oil. Fatty acids were methylated by 2% sodium methoxide to fatty acid

Table 4-3. Fatty acid components of transgenic seeds

Lines	**Fatty acid components (%)**							
	14:0	16:0	16:1	16:3	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9 Δ12
WS	0.073	7.266	0.260	0.069	3.573	15.783	1.496	28.849
92-5	0.071	6.428	0.186	0.071	3.167	16.776	0.017	28.515
92-6	0.072	6.475	0.203	0.128	3.223	16.619	1.282	27.678
92-13	0.071	**6.200**	0.170	0.074	2.969	16.497	1.218	27.852
92-14	0.070	6.273	0.181	0.116	3.042	16.300	1.179	28.061

Lines	**Fatty acid components (%)**						
	18:3 Δ9 Δ12 Δ15	20:0	20:1 Δ11	20:1 Δ13	22:1 Δ13	**Saturated**	**Unsaturated**
WS	14.871	2.415	21.327	1.832	1.970	13.327	86.457
92-5	16.252	2.239	22.174	1.712	2.141	11.905	87.844
92-6	15.971	2.267	21.814	1.712	2.195	12.037	87.602
92-13	**16.330**	2.184	22.274	1.759	2.291	**11.423**	**88.466**
92-14	16.041	2.194	22.269	1.710	2.211	11.580	88.069

methyl esters (FAMEs). Sample from each transgenic line and wild type control were analyzed by GC. Component of each peak was identified by comparing retention time of the standards and relative proportions of FAMEs were determined as percentages of summed peak areas. The results are given in Table 4-3.

The content of saturated fatty acids was slightly decreased in transgenic lines in comparison to the wild type control. The most noticeable decrease was 16:0 (Palmitate) of line 92-13, which was 1.066% lower than wild type. However the contents of several unsaturated fatty acids, especially 18:1 Δ9 (oleate), in transgenic lines were slightly increased. The most significant change was the 1.459% increase in 18:3 Δ9 Δ12 Δ15 (linolenate) in line 92-13. In conclusion, total percentages of saturated fatty acids were decreased in all the 4 transgenic lines, while the total percentages of unsaturated fatty acids were increased. Transgenic line 92-13 showed the highest change of 1.95% from saturated to unsaturated fatty acids. Presence of hydroxybutyryl-CoA (HB-CoA) in fatty acid-CoA (FA-CoA) pool might disturb the activity of acyltransferase, and result in changes of saturated and unsaturated fatty acid contents. Analysis of FA-CoA contents will show if the proportions of saturated and unsaturated FA-CoA as well as the presence of HB-CoA in the pool have changed. The trend of higher unsaturated and lower saturated fatty acids contents was observed in all 4 transgenic lines, which implied that production of PHB in transgenic seeds may help improve the seed lipid composition. This result suggested another benefit of introduction of PHB synthesis into plant seeds.

4.4 Future Work

#92phbB:phbC:pKYLX-NAP cytosolic construct was transformed into *Arabidopsis* via *Agrobacterium*-mediated floral dip transformation and 22 T_0 resistant plants were rescued. Among them, 17 lines were identified as having single copy transgenes determined by segregation ratios of Kanamycin resistant marker gene. In this study, the main focus was on the lines with single copy number, some of which were further characterized by molecular analysis.

Southern hybridization of the 4 selected homozygous lines encountered some difficulties and gave inconclusive results, possibly because of incomplete digestion of the genomic DNA. Although segregation ratio can be estimation for copy number, it would be more complicated to identify accurate copy numbers by using segregation ratio for multiple copy lines. Therefore it is also important to have Southern hybridization to obtain final results for identification of multiple copy gene insertions. If the secondary bands detected in the ^{32}P probe hybridization were really due to incomplete digestion of the genomic DNA, this problem could be resolved by decreasing genomic DNA and extending the digestion time. However, the signals of the hybridization probe would consequently be weak due to the less quantity of target genes.

Molecular analysis experiments should be performed in multiple copy lines as well. It would be interesting to compare expressions of the transgenes in single and multiple insertion lines. This could be done by real time RT-PCR, which can more accurately quantify differences in mRNA expression. The correlation of results of real time RT-PCR with the amount of PHB produced in seeds from each line would be interesting to study. The extraction and quantification of PHB is discussed in the following chapter.

It is also important to verify whether mRNAs have been correctly and efficiently translated to proteins. This can be done by Western blot analysis of proteins from developing seeds using antibodies obtained from immunization of recombinant proteins discussed in chapter 3.

4.5 References

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Chapter 5 PHB extraction and analysis

5.1 Introduction

5.1.1 Small scale PHB extraction and purification from transgenic seeds

The process of PHB extraction and purification from transgenic seeds is developed from the conventional method used in bacteria fermentation. This method is based on the solubility of PHB in chlorinated hydrocarbon solvents, such as chloroform, and its insolubility in methanol (Poirier 2001). Harvested plant seeds are crushed, so that solutions used in downstream procedures can pass through thick seed coats. Accumulated seed oils and pigments are removed from the crushed powder by hexane and methanol. PHB, which is in the powder, is extracted by solubilization in chloroform. Seed coat debris and other impurities are removed from the chloroform solution by filtration and water extraction. Purified PHB sample in chloroform is subsequently derivatized with sulfuric acid and methanol for GC analysis.

Two main difficulties of PHB extraction from plant seeds are: the presences of thick plant cell walls and seed coats, and a very low PHB yield produced in transgenic plants (Poirier 2001). The process of extraction of PHB and other types of PHA from transgenic plants is considered a foremost factor affecting production cost in term of mass production in crop plants. The process of extraction explained in this chapter is only feasible for laboratory scale experiments due to quantity of solvents used. Development of efficient methods of PHB and other types of PHA extractions from oilseeds still remain a challenging task. The goal of PHB extraction is not only to reduce the cost for the process, but also to recover other useful components such as oil, proteins and carbohydrates from the harvested seeds.

5.1.2 Summary

Mature transgenic seeds were harvested from homozygous lines of *Arabidopsis* T_2 plants, transformed with #92phbB:phbC:pKYLX-NAP, and used for PHB analysis. The seeds were ground until fine powder was obtained. PHB was extracted, purified and derivatized using a protocol modified from Riis and Mai (1988), and Slater et al. (1999). PHB samples from transgenic seeds, along with a negative control sample prepared from wild type seeds, were analyzed by gas chromatography (Slater et al. 1998). Percent yield of PHB produced in transgenic seeds was calculated utilizing a standard curve constructed from serial concentrations of HB monomer standards.

5.2 Materials and Methods

5.2.1 PHB extraction and purification from transgenic seeds

PHB was extracted using a protocol modified from Riis and Mai, (1988), and Slater et al. (1999). 500 mg of mature seeds were pooled from homozygous plants of transgenic lines with single copy transgene insert (lines 92-5, 92-6, 92-13 and 92-14). The seeds were ground to obtain fine powder with a mortar and pestle. Seed oil was removed by extraction twice with 20 mL hexane at 60°C for 2 hours. Seed sample was filtered, rinsed with fresh hexane, and air-dried for few hours. Other impurities were removed by extraction twice with 20 mL methanol at 60°C for 2 hours. The sample was filtered, rinsed with fresh methanol, and completely dried overnight at RT. Dried sample powder was dissolved in 4 mL chloroform, and heated in an oil bath to 100°C for 5 hours to solubilize PHB. After cooled down to RT, chloroform solution with PHB was transferred into a fresh tube using a pasture pipette to avoid impurities. Derivatization of PHB sample in chloroform was preformed by methanolysis and hydrolysis with methanol and sulfuric acid respectively, to obtain methylated HB methyl ester monomers. Methanol and sulfuric acid were added to a final ratio of 1 chloroform: 0.85 methanol: 0.15 sulfuric acid. The sample was heated in an oil bath to 100°C for 2.5 hours. One volume of distilled water was added to the sample and vortexed for 30 seconds to remove sulfuric acid and methanol. The organic phase, which contained HB methyl ester monomers in chloroform, was used for gas chromatography. A negative control sample was prepared from 500mg of wild type seeds following the same method.

5.2.2 Gas chromatography (GC) method for PHB analysis

Derivatized PHB was analyzed by gas chromatography (GC) following a method demonstrated by Slater et al. (1998). Methylbenzoate solution was prepared in chloroform to a final concentration of 10 mg/mL, and used as an internal standard. 0.2 mL methylbenzoate standard was added to 0.8 mL monomer HB sample extracted from mature seeds in a glass vial used for GC analysis. The final concentration of methylbenzoate standard was 2 mg/mL. The gas chromatograph used was a Varian GC3400 equipped with a Varian 8200 autosampler. 1 µL of PHB solution sample containing methylbenzoate internal standard was injected with a 1/10 split ratio into a DB-5 capillary column (0.25 mm by 25 m; 0.25 µm film thickness, J&W Scientific Inc., catalog #1225532, Folsom, CA). The flow rate of helium carrier gas was 22 mL/min with the total run time of 33 min. The initial column temperature of 60°C was held for 3 minutes, and then the temperature was increased by 10°C per min up to 240°C. Finally, the column temperature was raised by 30°C per min to 300°C and hold for 10 minutes. The injection was repeated three times to obtain average value.

5.2.3 Quantification of the extracted and purified PHB

Extracted and purified PHB was quantified using a standard curve constructed from relative peak areas of serial concentrations of HB methyl ester monomer standards. HB monomer standards were prepared from a commercial HB powder (Sigma-Aldrich Co., Oakville, ON) to a final concentration of 10 mg/mL in chloroform. HB solution was derivatized with the same method used for extracted PHB from transgenic seeds. Additional chloroform and 0.2 mL 10 mg/mL methylbenzoate were added to each sample of the PHB samples to make serial concentrations of 0, 0.5, 2, 4, 6 and 8 mg/mL. The final concentration of the methylbenzoate internal standard was 2 mg/mL. The samples were analyzed by GC using the same method as for transgenic seeds. Relative peak areas of PHB standards to methylbenzoate internal standard were used to draw a standard curve with a relation to HB methyl ester monomer concentrations. The standard curve was subsequently used to calculate the concentrations of the PHB in transgenic seeds.

5.3 Results and Discussion

5.3.1 HB standard curve

HB methyl ester monomer standard samples prepared from a commercial HB powder (Sigma) with methylbenzoate standard in chloroform (0.5, 2, 4, 6, 8 mg/mL) were injected into gas chromatograph. The injection was repeated three times with an injection of blank chloroform in between samples of different concentrations. Each of the chromatograms of the samples showed two peaks at identical retention times of 2.96 ± 0.01 minutes and 7.44 ± 0.01 minutes (Figure 5-1). To identify the peaks, HB methyl ester monomers in chloroform solution and methylbenzoate were analyzed separately using the same method. The HB methyl ester revealed a peak at retention time 2.96 minutes, while the methylbenzoate showed a peak at retention time 7.44 minutes. Relative retention time between HB sample and methylbenzoate was 4.485 ± 0.01 minutes.

For proper quantification, relative peak areas of HB samples were calculated relatively to methylbenzoate internal standard peak area, which has the same concentration in every sample (2 mg/mL. Average values of relative peak areas calculated from three repetitions were used to draw the standard curve. The HB standard showed a linear relation between the relative peak areas and the concentration of HB monomers (mg/mL) corresponding to "Relative peak area = 0.026 concentration (mg/mL) – 0.0164" equation with an R-squared value of 0.9954.

5.3.2 Extracted PHB from transgenic seeds

Mature *Arabidopsis* seeds were harvested from homozygous plants from lines 5, 6, 13 and 14 and pooled to obtain 500 mg seeds. The seeds were crushed to obtain fine powder with mortar and pestle. PHB was extracted and derivatized as described in 5.2.1. The same extraction and purification process was performed using mature wild type seeds, and the sample was used as a negative control for this experiment. Two sets of HB monomer samples extracted and purified from transgenic and wild type seeds were injected into the gas chromatograph using the same GC program as for HB standards.

Each set was run for 3 times.

GC analysis results of samples extracted from transgenic and wild type seeds are shown in Figure 5-2. Chromatograms from three injections of two sets of transgenic sample showed a small peak at retention time 2.985 ± 0.005 minutes corresponding to the retention time of HB monomer standards, while the wild type control did not show any detectable signal around this retention time in any injection. Both wild type and transgenic samples showed signals of methylbenzoate internal standard at 7.475 ± 0.005 minutes. Relative retention time of the internal standard of two transgenic HB monomers was 4.492 ± 0.01 minutes corresponding to the number obtained from HB monomer samples used to draw the standard curve. The tolerance value of the relative retention time obtained was 0.2%, which is qualified for GC analysis. Average relative peak areas of the two sets of transgenic HB were used to calculate concentrations of based on the linear relation between relative peak areas and HB concentrations of the standard curve. Concentration of HB of seed sample set 1 was quantified 0.775 mg/mL and HB concentration of seed sample set 2 was quantified 0.768 mg/mL.

However, only 0.8 mL of HB samples extracted from transgenic seeds were used to prepare 1 mL of GC sample. Thus the original concentrations of HB in chloroform from two sets of the seeds were 0.97 mg/mL and 0.96 mg/mL with a total amount dissolved in 4 mL chloroform. These numbers indicated that the initial amounts of PHB in 500 mg of raw seed material, which was extracted and purified in 4mL of chloroform, were 3.88 mg in the first set and 3.84 mg in the seeds. As a result the average yield of PHB produced in mature transgenic seeds from two sets of experiments was 0.772 ± 0.006 % of seed weight.

A similar attempt was carried out in the past with separate transformations of *phbB* and *phbC* genes from *R. eutropha* in *Arabidopsis*, each under control of a constitutive cauliflower mosaic virus 35S promoter (CaMV35S) (Poirier et al. 1992). Presence of granules, similar to PHB granules found in natural producer bacterial cells, was observed in leaf mesophyll cells under transmission electron microscope. However identification of the granules as PHB failed due to an extremely low amount of PHB accumulated in transgenic plants. To increase production of PHB enough for extraction and analysis, an *in vitro* cell suspension culture was performed with transgenic PHB-producing *Arabidopsis* plant cells (Poirier and Somerville 1995). About 500 µg of PHB was obtained from 1 g of cell suspension by chloroform extraction, which indicated a yield of 0.05 % of cell suspension fresh weight. PHB extracted from 100 g of cell suspension was sufficient for extraction and GC analysis. The PHB-like granules accumulated in transgenic *Arabidopsis* leaves were confirmed to have the same retention time with PHB from *R. eutropha.*

In this study, a strong seed-specific Napin promoter was used instead of constitutive CaMV35S promoters, and two transgenes, *phbB* and *phbC*, were prepared in one double-cassette gene construct. Expressions of the two transgenes were exclusively directed and enhanced into developing seeds with an expectation to increase production of PHB. Seeds are the main compartments of plant in which oil accumulation takes place. Thus in developing seeds, there is a large pool of acetyl-CoA, which can be utilized for PHB synthesis as well. The average PHB yield obtained from transgenic lines in this study was 0.772 ± 0.006 % of seed weight, which is a dramatic increase of 15.5-fold higher compared to the previous attempt (0.05%). From these results, the strategy of using seed-specific promoters to direct PHB synthesis utterly into developing seeds, combined with a single transformation with one double-cassette construct was successfully demonstrated in the present study.

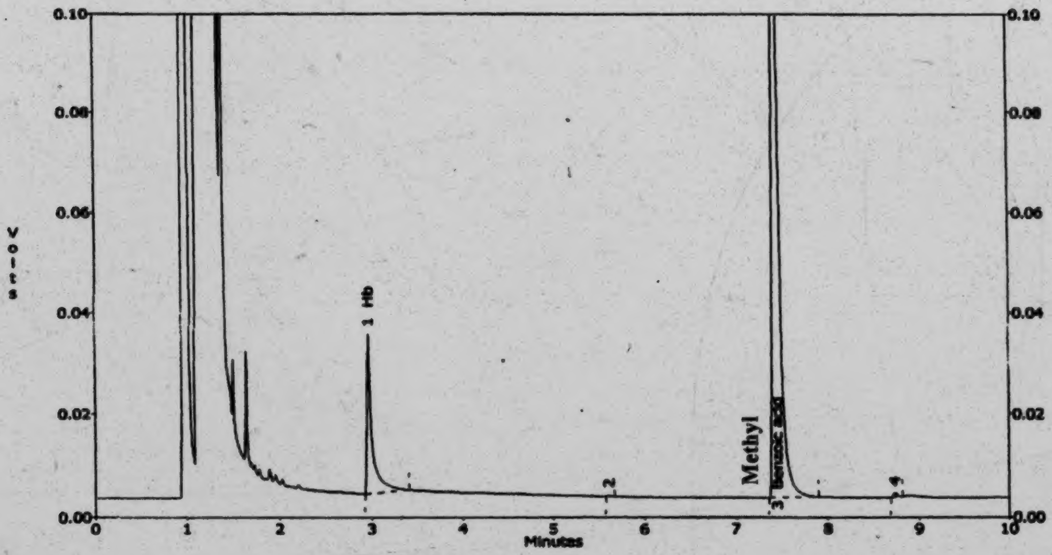
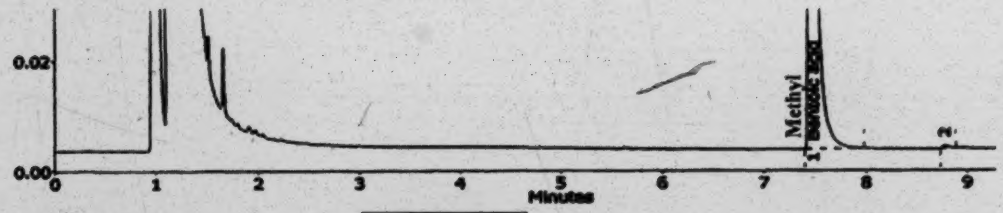


Figure 5-1. Chromatogram of HB monomer standard with methylbenzoate internal standard from Gas Chromatography
The sample contains HB monomer (4 mg/mL) and methylbenzoate internal standard (2 mg/mL), which show peaks at retention times of 2.96 and 7.44 minutes, respectively. The relative peak area of HB monomer to methylbenzoate standard was used to construct the HB standard curve.

a. wild type



b. transgenic

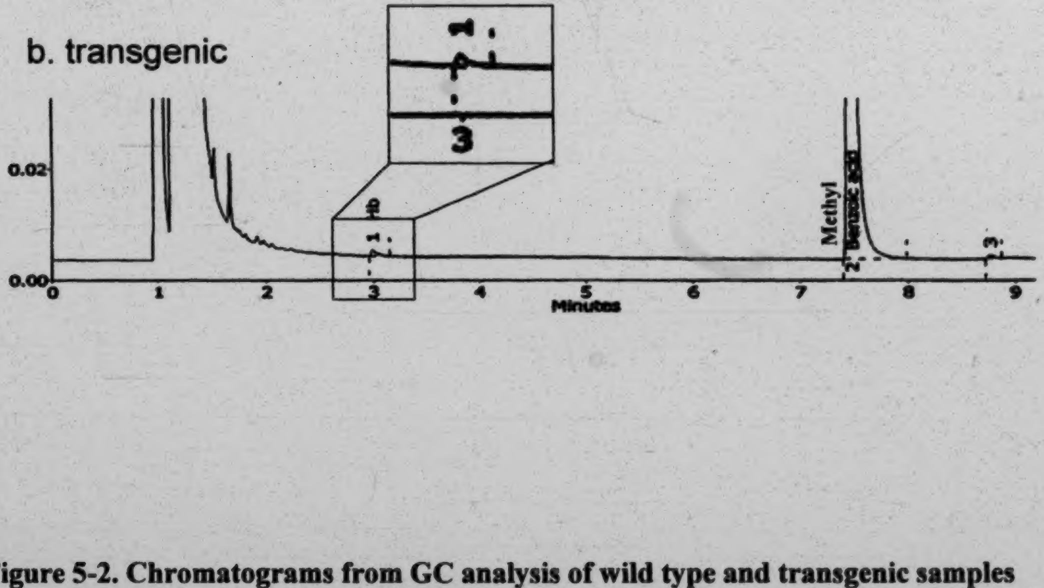


Figure 5-2. Chromatograms from GC analysis of wild type and transgenic samples
a.) Sample from wild type seeds (negative control) shows only one peak of methyl benzoate internal standard at retention time of 7.45 minutes. b.) Sample from transgenic seeds shows a small peak of HB monomer at retention time of 2.97 minutes and a peak of methylbenzoate at 7.44 minutes.

5.4 Future Work

In this study, PHB produced in the transgenic *Arabidopsis* seeds was successfully extracted and purified. The average yield of accumulated PHB was determined by GC analysis as 0.772 ± 0.006 % of seed weight. Mature seeds were harvested and pooled together from 4 homozygous transgenic lines (lines 5, 6, 13 and 14) to bulk up the amount of the seeds to 1 g to be sufficient for two sets of extraction and analysis. Therefore, high PHB yield in any of the 4 lines has been flattened to average value by the low yielders. Even though all 4 transgenic lines were obtained from transformation of #92phbB:phbC:pKYLX-NAP, they were derived from independent T_0 transformants. The positions in which transgenes have been integrated into plant genomes in each line were unspecific and expected to be diverse. To investigate positional effect of the transgenes on PHB production, sufficient transgenic seeds should be produced from each transgenic line to perform PHB extraction and analysis. Lines with high PHB production yield can be selected for a mass production and further development.

It is also interesting to examine in which cellular compartments PHB synthesis and accumulation have taken place, as well as size of PHB inclusions in transgenic seeds compared to those synthesized by a natural producer. Since the two transgenes, which were integrated into plant genome, have no organelle-specific signal attached, the accumulation of PHB is expected to take place in only cytoplasm. Furthermore in natural producers, such as *R. eutropha,* a phasin protein called PhbP was naturally found. PhbP has a function of increasing surfaces of PHB granules by preventing formation of large PHB granules. A *phbP* mutant strain of *R. eutropha* was reported to accumulate only a single PHB granule, while the wild type strain accumulated many small PHB granules (Madison and Huisman 1999). Therefore the size of PHB accumulated in transgenic seeds, in which no expression of phasin proteins exist, is expected to be larger than PHB granules produced by natural producers. These two aspects of localization and size of PHB granules can be studied by observation of a cross-section of developing seed tissue under a transmission electron microscope.

Although only 4 homozygous lines, which had good growth condition and seed productivity, were analyzed, many other lines were generated during this study. It is possible that lines with poor seed production and germination might have much higher yield of PHB. Therefore it is also important to develop extraction and analysis method that requires a small amount of seeds.

5.5 References

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Chapter 6 General Discussion and Conclusion

6.1 Recapitulation of research objectives and main findings

The study described in this thesis illustrates creation of transgenic *Arabidopsis* that produces Polyhydroxybutyrates (PHB) in the cytosol of developing seeds. PHB is the most common and extensively studied type of Polyhydroxyalkanoate (PHA), which belongs to the biodegradable polyester family. PHB is naturally accumulated in *Ralstronia eutropha* cells as an energy storage molecule to be metabolized when other common energy sources are not available (Madison and Huisman 1999). The main substrate of PHB biosynthesis is acetyl-CoA, which is also found ubiquitously in cytoplasm and plastids of higher plants. In developing seeds, where fatty acid synthesis takes place, a large pool of acetyl-CoA is present because it is the major substrate for fatty acid synthesis. In this study the PHB synthesis pathway was introduced into *Arabidopsis* developing seeds by expressing *phb* genes from *R. eutropha* under control of seed specific Napin promoter. Mature transgenic seeds harvested from homozygous lines accumulated PHB with the yield of 0.772% of seed weight.

A first step that had to be taken in order to introduce PHB synthesis pathway into developing seeds was the development of plant expression gene constructs (Chapter 2). Three genes, which encode three enzymes responsible for PHB synthesis, were isolated from *R. eutropha*. The gene constructs developed in this study were designed for expressions in cytosolic and plastidial compartments. It was discussed in Chapter 1 and Chapter 2 that production of PHB in the cytosolic compartment requires only expression of two transgenes, *phbB and phbC*, since the first enzyme of the synthesis pathway, β-ketothiolase, is naturally found in plant cytosol as a part of the isoprenoids production (Moire et al. 2003). Therefore, a *phbB* and *phbC* double-cassette construct driven by seed-specific Napin promoter with Kanamycin resistant marker was prepared. Although this study was focused on introduction of PHB synthesis pathway into cytoplasm of developing seeds, gene constructs for PHB production in plastids were also developed for future research. The production of PHB in plastid requires expression of all three transgenes (*phbA*, *phbB* and *phbC*) as well as an addition of chloroplast transit peptide (CTP) to each of them, to direct the expression of the enzymes into the plastids (Nawrath et al. 1994). The CTP sequence was isolated from *Arabidopsis* genomic DNA and utilized in targeting a protein expression into the plastid (Corbin et al., 2001). Two gene constructs were prepared with different selectable markers, Kanamycin and PPT herbicide resistance, to facilitate co-transformation. One with Kanamycin resistance marker was a *phbA* and *phbB* double-cassette construct driven by seed-specific promoters with CTP target signal sequences. The other with PPT herbicide resistance marker, a *phbC* single gene construct, was also driven by a seed specific promoter with CTP sequence (described in Chapter 2).

In the second step, the *phbB* and *phbC* double-cassette construct with Kanamycin resistance marker was introduced into *Arabidopsis* plants via *Agrobacterium*-mediated floral dip transformation (described in Chapter 4). *Agrobacterium* has the capability to transmit T-DNA into plant genome, which has been extensively utilized to insert foreign genes in plants through genetic engineering (Zambryski 1983). Transformed plants carrying the gene construct were screened from seedlings by germinating the T_0 seeds on 1/2MS plates with Kanamycin selection. Twenty-two transformed plants were obtained and copy numbers of inserted genes in them were identified by segregation ratio of Kanamycin resistant progenies from each T_0 parental lines. Seventeen lines exhibited segregation ratio close to 3 (resistant):1(susceptible), which follows the ratio from self-pollination of a heterozygous monohybrid parent (Aa), and were therefore predicted to have single copy transgenes. The remaining 5 lines, which exhibited segregation ratios closer to or more than 15(resistant): 1(susceptible), were predicted to have two or more copies of transgenes. In this study, the main interest was focused on lines with single copy number, which were continued further for molecular analysis.

Genomic DNA extracted from T_1 plants of 7 healthy lines were used to confirm the integration of transgenes into plant genome by PCR method (Kasajima et al. 2004). PCR reactions were performed using internal primers of *phbB* and *phbC*. All the individual plants were confirmed to carry both transgenes, whereas no positive PCR product was found in the wild type genome. Among those 7 lines, 4 lines with healthy growth and good seed productivity were continued for further analysis. Homozygous lines were developed from them and southern hybridization and reverse transcription-PCR were performed to confirm the copy number and expression of the transgenes, respectively. T_2 seeds harvested from individual Kanamycin resistant plants of each of the 4 lines were germinated on 1/2MS plates with Kanamycin selection to determine homozygousity. A few T_1 plants from each line revealed homozygousity with all resistant progenies. The others exhibited heterozygousity with progenies segregating as 3(resistant): 1(susceptible), which once more confirmed a single copy transgenes in these lines. Southern hybridization utilizing genomic DNA from homozygous plant was undertaken to confirm the copy numbers of the transgenes. The problems encountered were possibly incomplete digestion of genomic DNA and signal detection of hybridized bands (Chapter 4). The analysis of transcription of the transgenes into mRNAs in the developing seeds was performed by RT-PCR. Total RNA was extracted from immature seedpods (including developing seeds) collected and pooled from homozygous plants. RT-PCR reactions were performed using primers for amplifications of the full-length *phbB* and *phbC* genes. Amplified RT-PCR products of transgenic materials showed correct bands with expected sizes, while wild type negative control did not give a positive band. From these results, the transcriptions of *phbB* and *phbC* genes in developing seeds were confirmed (Chapter 4). Since Acetyl-CoA is a substrate in both PHB and fatty acids biosynthesis pathways, fatty acids compositions of transgenic seeds were investigated as a comparison to those of wild type seeds. Results from GC showed higher unsaturated and lower saturated fatty acids contents in all 4 transgenic lines. This trend implied improvement of the seed lipid compositions in transgenic lines (Chapter 4)

The expression of recombinant proteins, encoded by the transgenes, in developing seeds can be identified by Western hybridization with the anti-Phb antibodies. To raise anti-Phb antibodies, *phb* genes were inserted into protein expression vectors for over expression in *E. coli* and recombinant proteins were purified (PhbA, PhbB and PhbC) (described in Chapter 3). Purified Phb proteins were used for immunization of rabbits for polyclonal antibodies production. These antibodies can be used in Western hybridization to further analyze the transgenic plants in the future. The target proteins were produced with an attachment of N-terminal His-tag to facilitate column purification of recombinant proteins. His-tagged Phb proteins were over-expressed in protein expression host, *E. coli* BL21(DE3) with IPTG induction. The expression level, molecular weight, and solubility of each protein were determined by SDS-PAGE, and western hybridization with anti-His western antibody. Productions of recombinant proteins were scaled up and recombinant PhbA and PhbB were successfully purified by passing the cell lysate through a Ni-column. Attempts to purify PhbC protein were carried out under both native and denaturing conditions, and encountered some difficulties. The major problem was caused by insolubility of PhbC protein itself. PhbC is a granule-associated protein that forms inclusion bodies attached to the cell membrane at certain saturation point (Haywood et al. 1989). Purified PhbA and PhbB samples were quantified following a modified Bradford assay (Bradford 1976) with BSA as the standard. Purified PhbA and PhbB samples were sent to University of Calgary SACRI Antibody Services for immunization into rabbit hosts for the productions of polyclonal antibodies (Chapter 3).

Mature transgenic seeds from homozygous lines with a single copy cytosolic double-cassette transgenes were utilized for PHB extraction and analysis (Chapter 5). PHB was extracted and purified from 500 mg seeds, using a protocol adapted from Slater et al. (1999) and Riis and Mai (1988). PHB samples were derivatized to HB monomers by addition of sulfuric acid and methanol, and were analyzed by GC as described by Slater et al. (1998). Transgenic sample showed a small HB methyl ester peak at retention time of 2.985 ± 0.005 minutes that corresponds to the retention time of HB methyl ester monomer standards. The wild type control did not show any detectable signal. Standard curve of HB monomers was constructed using peak areas obtained from serial concentrations of the HB standard. Percent yield of PHB in transgenic seeds was calculated based on the standard curve to be 0.772 ± 0.006% of seed weight (Chapter 5). This yield represents 15.5-fold increase compared to a similar work previously done by Poirier and Somerville (1995), which obtained 0.05 % PHB in cell suspension fresh weight.

In this study there were two main strategies that were successfully modified. First, seed-specific Napin promoter was used instead of CaMV35S constitutive promoters. Seeds are the main compartments of plant in which oil accumulation takes place. Thus in developing seeds there are a large pool of acetyl-CoA, which can be utilized for PHB synthesis as well. Second, the use of a double-cassette construct for a single transformation instead of the cross pollination of two transgenic plants, which carries different gene constructs.

6.2 Suggestions for further research

The overall result of the present study was a dramatic 15.5-fold increase of PHB synthesis in cytosol compared to the similar work previously done by Poirier and Somerville 1995. The key strategies of this improvement were the uses of seed-specific Napin promoter and a double-cassette construct for single transformation of two transgenes. Even though the yield obtained was 0.772% of seed weight, this number was still incomparable to percentage of oil accumulation in the seeds. The yield of 0.772% was approximately 60 times lower than oil accumulation in mature *Arabidopsis* seeds (40% of seed dry weight) (Poirier 2001). Synthesis of fatty acids takes place in plastids of developing seeds, also referred to as leucoplasts. The synthesis of fatty acids utilizes acetyl-CoA as a precursor, which is also used as a substrate for PHB production. Therefore, leucoplast is a site where a high flux of acetyl-CoA is found. Consequently introduction of PHB synthesis into leucoplast of developing seeds is expected to give a significantly higher yield of PHB. Therefore, plastidial gene constructs driven by seed-specific promoters developed in this study (Chapter2) are suggested to be co-transformed into plants to introduce PHB synthesis into leucoplast. For mass production of PHB, *Arabidopsis* may not be an appropriate candidate. Since acetyl-CoA is a central substrate for both PHB and fatty acid biosynthesis, oil-seed crops such as *Linum usitatissimum* L. (flax), which is efficient in oil production with large substrate pool, represents a more attractive candidate. Both cytosolic and plastidial gene constructs are currently introduced into *L. usitatissimum* L. (data not shown). It would be interesting to compare the yield of PHB in transgenic seeds of *Arabidopsis* and *L. usitatissimum* L., transformed with the same gene constructs.

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